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LACTIC OXIDSES OF MYCOBACTERIUM TUBERCULOSIS AVIUM

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(Received for publication, December 15, 1951)

Yamamura *et al.* carried out the studies on the metabolism of *Mycobacteria*, especially of the tuberculous bacillus. Recently we have demonstrated that the cell-free enzyme preparations catalyzing only the oxidation of lactate (*1*) can be obtained from *Mycobacterium tuberculosis avium*. It was confirmed that these enzyme preparations involved two distinct lactic oxidases; namely, one reacted directly with molecular oxygen without the mediation of hydrogen carrier, whereas the other required the addition of hydrogen carrier such as methylene blue (*2*). Among them, the former was partly purified and it was found to be quite different from the lactic dehydrogenases of animal tissues (*3*), yeast (*4*), *E. coli* (*5*), *Gonococcus* (*6*), and higher plants (*7*). It is the purpose of the present paper to describe especially the properties of this enzyme.

EXPERIMENTAL

Methods:

Enzyme Activity—The oxidation of lactate was estimated by measuring the oxygen uptake manometrically in a Warburg apparatus at 37°. In most of the experiments, 0.3 ml. of 0.2 *M* phosphate buffer (pH 6.2), 2 ml. of enzyme preparation, and 0.2 ml. of 3 *M* DL-lactate were placed in the main chamber, and 0.3 ml. of 20 per cent potassium hydroxide in the central well.

Estimations—Lactate was determined manometrically with ceric sulfate (*8*). Volatile acids were estimated by distillation and titration (9). Volatile acids were estimated by distillation and titration (9). Pyruvate was estimated by the bisulfite-binding method of Clift and Cook (*10*) and ceric sulfate method (*11*).

Materials—Diphosphopyridine nucleotide (DNP), adenosine triphosphate (ATP), riboflavine phosphate (FMN), catalase, cytochrome c, cytochrome

c oxidase and the apoprotein of D-amino acid oxidase were prepared according to the method of Williamson and Green (*12*), LePage (*13*), Kuhn *et al.* (*14*), Sumner (*15*), Keilin and Hartree (*16*), Keilin and Hartree (*17*), and Warburg and Christian (*18*), respectively.

Flavinadenine dinucleotide (FAD) was kindly supplied by Prof. Kubo and Dr. Yamano of the Medical School of the University of Osaka.

Growth of Bacteria—Mycobacterium tuberculosis avjum, strain Takeo, was used. The composition of the culture medium was as follows: fish (bonito) extract 20 g., peptone 20 g., NaCl 2 g., glycerol 50 ml., and distilled water 1000 ml. The bacteria were usually grown in six or ten 300 ml. of cultures simultaneously for 3 days at 37° and were harvested together.

Extraction and Purification of Enzyme—After washing the cells with distilled water, the compact mass of bacteria was rapidly frozen, allowed to stand at—10° for 24 hours and then thawed by incubating at 37°. The bacteria were suspended in 0.04 *M* phosphate buffer (pH 7.5), and the thick suspension was frozen again, allowed to stand at—10° for 24 hours and thawed at 37°. After standing at 0° for 2 daps the suspension was centrifuged at 10,000 r.p.m. for 20 minutes. The supernatant solution (A) was adjusted to pH 3.8 with 10 per cent acetic acid and centrifuged immediately. The precipitate was placed in a desiccator over H_2SO_4 and dried *in vacuo* at room temperature. The precipitate which not used immediately could be stored under vacuum at 0° for 2 weeks without appreciable loss in activity. The dired precipitate was suspended in a small amount of 0.2*M* phosphate buffer (pH 6.2). kept for 60 to 90 minutes at 0° and centrifuged. Then the precipitate was discarded, and 22 g. of ammoniun sulfate were added per 100 ml. of the supernatant solution. The obtained solution was refrigerated for 1 hour, centrifuged, and the precipitate was discarded. To the supernatant 22 g of ammonium sulfate were added to bring the solution to saturation. It was kept at 0° overnight, and centrifuged on the next day. The precipitate was taken up in a small volume of 0.2 *M* phosphate buffer (pH 6.2) and the residue removed by centrifugation. The supernatant (B) was dialyzed for 2 days against ice-cold distilled water and, thereafter, adjusted to pH 4.5 with 10 per cent acetic acid. Again the formed precipitate was dried *in vacuo*, extracted with 0.2 *M* phosphate buffer (pH 6.2), and the residue discarded. The fractionation with anmonium sulfate was repeated. The resulting solution was dialyzed for 2 days against ice-cold distilled water (G). Sometimes the third fraction with ammonium sulfate was also prepared. The purified preparation (B and C)

stored at 0° showed no appreciable loss in activity over a period of week. Attemps to purify the enzyme by adsorption and elution from calcium phosphate gel, Cγ and caolin were unsuccessful. The uses of alcohol and acetone destroyed about 100 and 80 per cent of the enzymic activity, respectively. The extraction was also made possible by autolysis of acetone-powder of bacteria. But the resulting extract contained much more methylene blue-linked lactic enzyme than the extract by alternative freezing and thawing method, and very often non methylene blue-linked enzyme, which is to be described here in more details, was of little activity.

RESULTS

Occurrence of Two Lactic Oxidases in the Crude Extract—The crude cell-free enzyme preparation (A) was able to oxidize lactate without the addition of methylene blue. However, the addition of methylnene blue increased the consumption of oxygen as much as by 30 to 50 per cent (sometimes even over 100 per cent) (Fig. 1).

When the extract was heated for 10 minutes at 50°, its capacity to oxidize lactate was almost completely lost. When, however, methylene blue was added to such previously heated extract, its capacity was not so much lowered compared with that of unheated extract (Table 1). On the other hand, dialysis against water at 0° for 12 hours had no effect on the oxidation of lactate in the absence of methylene blue, but it abolished only the effect of methylene blue. (Table I)

These phenomena can be explained as follows: There are two distinct lactic oxidases, one being heat-labile, dialysis-stable and capable of reacting directly with molecular oxygen; and the other being relatively heat-

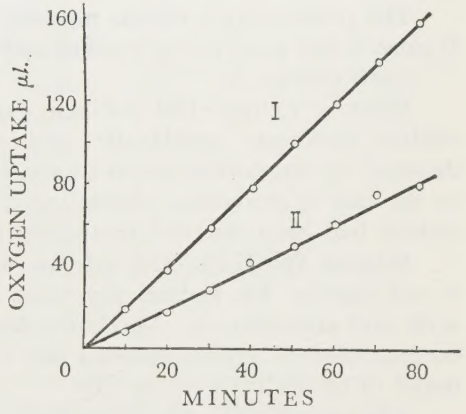


Fig. 1. The effect of methylene blue on lactic oxidation by the crude enzyme preparation.

Warburg vessels contain 2.0 ml. of crude extract (A) obtained by alternative freezing and thawing, 0.3 ml. of 0.2 *M* phosphate buffer(pH 6.2), and 0.2 ml. of 3 *M* DL-lactate. Gurve I, 500 γ of methylene blue was added. Curve II, no addition.

TABLE I

The Effect of Heating and Didlysis on Lactic Oxidation by Crude Enzyme Preparation

Preparation	Oxygen uptake per 60 min.	
	Witout methylene blue	With methylene blue
	μl.	*μl.*
Original (A)	101	210
Heated for 10 min. at 50°	0	125
Dialyzed for 12 hrs. at 0°	109	94

All conditions were the same as for Curves I and II in Fig. 1, respectively.

labile, and active only in the presence of methylene blue or other sorts of redox-dye. For convenience the former is called "Lactic oxidase I" and the latter is referred to as "Lactic oxidase II" in the following.

The present paper reports especially the properties of Lactic oxidase I, since it has been partly purified and concentrated.

Lactic Oxidase I:

Effect of Carrier—The purified enzyme preparations (B and C) can oxidize DL-lactate aerobically and the addition of methylene blue, thionine, or cytochrome c-cytochrome oxidase system gave no effect at all on the rate of oxidation. Accordingly, it seemed that lactic oxidase II activity had been removed completely in the course of purification.

Substrate Specificity—This enzyme was able to oxidize only DL-lactate; it was inactive for malate, pyruvate, acetate, succinate, formete, amino acids and acetaldehyde. Maximum activity was attained at a substrate concentration of approximately 1 M. The Michaelis-Menten constant was found to be 5×10^{-2} *mole per liter.*

Effect of pH—The rate of oxidation was essentially the same over the pH range form 5.5 to 8.5. The optimum pH was about 6.2.

Effect of Inhibitors—Table II illustrates the effects of various inhibitors. The lack of cyanide, azide and 8-hydroxy-quinoline inhibition suggests the absence of a heavy metal component. The complete inhibitions by silver nitrate and copper sulfate are similar to the properties of the L-amino acid oxidase of *Proteus vulgaris* reported by Stumpf and Green (*19*).

Prosthetic Group—Additions of various coenzymes (DPN, ATP, FAD, FMN and Mg^{++}) gave no effect on the enzyme. All autempts to resolve

the enzyme into native apoenzyme and prosthetic group failed, at least in working with crude or purified preparations. Attempts at resolution included prolonged dialysis, treatment with HCI and ammonium sulfate (*18*), and with acetone, adsorption and heating.

This enzyme had not any readily dissociable cofactors. Sometimes, however, the oxidation rate of lactate had been slightly stimulated by the addition of FAD, but not by FMN. (Table III)

Since flavoproteins are known to react directly with molecular oxygen, attemps were made to detect flavins in the enzyme preparations. These enzyme preparations were heated at 85° for 15 minutes and the precipitates

TABLE II

The Effect of Inhibitors

Inhibitor	Final concentration	Inhibition
	M	*per cent*
Silver nitrate	1×10^{-4}	100
Copper sulfate	3.3×10^{-4}	100
8-Hydroxy quinoline	1.3×10^{-4}	0
Cyanide	4×10^{-3}	0
Azide	4×10^{-3}	0
Iodoacetate	4×10^{-3}	0
Arsenite	2.5×10^{-3}	0
Fluoride	8×10^{-3}	0
Pyruvate	5×10^{-3}	0
Malonate	4×10^{-2}	0

TABLE III

The Effects of FAD and FMN

Addition	Oxygen uptake per 180 min.	
	Preparation I	Preparation II
	μl.	*μl.*
No addition	214	168
FAD (20 γ)	347	249
FMN (100 γ)	247	—

Preparation I was prepared by dialyzing B for 5 hours. preparation II was prepared by precipitating B with cold acetone. The same data have been obtained only several times.

were removed by centrifugation. The clear superantant fluids were added to the mixture consisting of the apoprotein of pig kidney D-amino acid oxidase (*18*) and DL-alanine, and then the oxidation rates of DL-alanine were determined. The oxidation rate of alanine due to each enzyme preparation (FAD activity,) increased remarkably with the increasing degree of purification. Tables IV and V show an example of such experiments.

Inasmuch as FAD is the only substance known to activate D-amino acid oxidase, these experiments seem to suggest that the prosthetic group of this enzyme is FAD. However, a good deal experimental work will have to be done before the final conclusion on the nature of the prosthetic group can be made.

Effect of Inorganic Phosphate—In order to detect the effect of inorganic phosphate, the enzyme was extracted by using veronal-HCI bufler instead of phosphate buffer. Inorganic phosphate had no influence on the lactic oxidation of this enzyme preparation.

Catalyzed Reaction—In the presence of lactate the equivalent of 1 mole of oxygen per mole of substrate was used. Pyruvate was not detectcd in reaction product, while the equivalent of each 1 mole of carbon dioxide and volatile acid was formed per mole of substrate (Table VI).

The reaction catalyzed by this enzyme can be expressed by the following equation:

$$CH_2CHOHCOOH + O_2 \text{——} CH_3COOH + CO_2 + H_2O \ldots\ldots (1)$$

The enzyme does not attack pyruvate, whether cocarboxylase is present or not. It will be concluded that the enzyme catalyzes the oxidation of lactate acetate and carbon dioxide without intervention of pyruvate.

Absence of Hydrogen Peroxide—The possibility of hydrogen peroxide formation during the oxidation was ruled out from the following experiments: (a) The preparations (evenC) still contained catalase activity as an impurity. The addition of alcohol in an attempt to obtain the coupled oxidation had no effect on Qc_2 or on the total oxygen consumption; (b) Catalase was completely inhibited by azide or cyanide. The addition of azide or cyanide in an attempt to prevent the action of catalase on the lactic oxidation gave no effect on Qo_2 or the total oxygen consumption; (c) Neither the addition of hydrogen peroxide nor the addition of active beef liver catalase showed any effect.

Lactic Oxidase II:

While the extract (A) obtained by alternative freezing and thawing showed much less activity of lactic oxidase II than that of lactic oxidase

TABLE IV

Qo_2 and FAD Activity of Enzyme Preparation

Enzyme	Substrate	Buffer	Addition	Oxygen uptake per 60 min.	Qo_2 and FAD activity†
Lactic oxicase B preparation	DL-Lactate	0.3ml. of 0.2 *M* phosphate	No addition	*μl.* 498	150*
Apoprotein of D-amino acid oxidase	DL-Alanine	1.0ml. hf 0.1*M* pyrophosphate	No addition	0	
,,	,,	,,	FAD (5 γ)	323	
,,	,,	,,	Heated lactic oxidase I (B)	109	50+

TABLE V

Qo_2 and FAD Activity per mg. of Enzyme Preparations

Nnzyme preparation	Qo_2*	FAD activity†
A	15	17
B	150	50
C	450	150

* *μl.* of O_2 per mg. of dry weight of enzyme per 60 minutes.

† *μl.* of O_2 per mg. of dry weight of heated lactic oxidase used instead of FAD per 60 minutes. The authors define this value as FAD activity.

TABLE VI

Chemical Balance of Lactic Oxidation

Experiment No.	Consumption		Formation	
	Oxygen	DL-Lactate	Carbon dioxide	Volatile acid
	micromoles	*micromoles*	*micromoles*	*micromoles*
I	17.6	17.6		12.8
II	71.2		74.0	68.8

I, the acetone-powder extract contained comparatively more activity of lactic oxidase II. Some of the preparations, showed remarkable acceleration of lactate oxidation on adding of methylene blue,.

It appeared that latic oxidase II was obtained in a good yield when the organism was grown on a low content of glycerol.

Properties of Oxidase II—Attempts to purify and concentrate the enzyme have not yet beed successful. Accordingly, for the study the extract of acetone-powder was employed, although it contained a certain amount of lactic oxidase I. Table VII shows the activation by methylene blue, cytochrome c-cytochrome oxidase system, or DPN.

TABLE VII

Effect of Methylene Blue, Cytochrome c-Cytochrome Oxidase, and DPN on Lactic Oxidation by the Extract of Acetome-Powder.

Addition	Oxygen uptake per 60 min.	
	Experiment I	Experiment II
	μl.	*μl.*
No addition	70	14
Methylene blue	153	54
DPN	106	
Methylene blue and DPN	252	90
Cytochrome c		17
Cytochrome oxidase		131
Cytrchrome c and cytochrome oxidase		172

In the presence of methylene blue, pyruvate was formed as the reaction product (Table VIII).

Assuming that the oxygen consumption in the absence of methylene blue was due to lactic oxidase I, while the increase of oxygen consumption in the presence of methylene blue was due to latic oxidase II. Table VIII suggests the following reaction catalyzed by the latter.

$$CH_3CHOHCOOH+\tfrac{1}{2}O_2 \longrightarrow CH_3COCOOH+H_2O \quad \text{.....(2)}$$

DISCUSSION

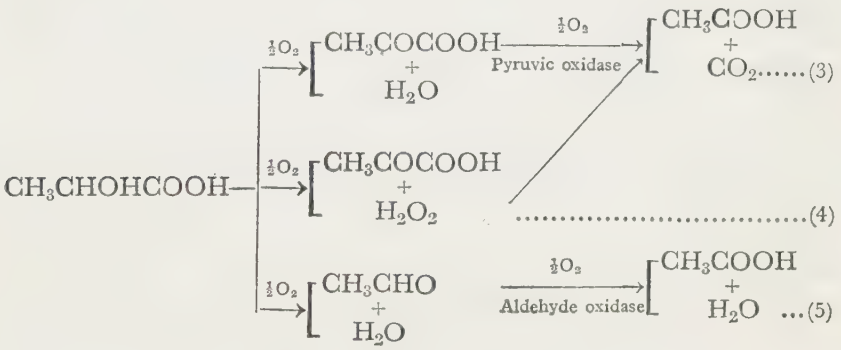
Lactic oxidase I differs apparently from the lactic dehydrogenases of animal tissues (*3*), yeast (*4*), *Gonococcus* (*6*), *E. coli* (*5*), and higher plants (*7*), which all catalyze the oxidation of lactate to pyruvate. The

TABLE VIII

Formation of Pyruvate

Addition	Oxygen uptake per 210 min.	Pyruvate formation per 210 min.
	micromoles	*micromoles*
Lactate	57	0
Lactate and methylene blue (500 γ)	104	50

cation of this enzyme is not simple dehydrogenation, but oxidative decarboxylation similar to pyruvic oxidase rather than lactic dehydrogenases reported hitherto. The mechanisms of lactic oxidation by the previously reported enzymes are as follows:



Since the enzyme attacks neither pyruvate nor acetaldehyde, reactions (3) and (5) are excluded. Reaction (4) is probably either spontaneous or is catalyzed by the coupled oxidation of catalase, and these possibilities are ruled out from the experiments previously described. And still the author can not conclude the real mechanism of the lactic oxidation by this enzyme. On the other hand, lactic oxidase II is very similar to the lactic dehydrogenase of animal tissues.

Edson obtained a lactic oxidase from *Mycobacterium phlei* (*20*). This enzyme was cyan-insensitive, FAD-linked and catalyzed the oxidtion of lactate to acetate aerobically, while anaerobically to pyruvate in the presence of methylene blue (*21*). It seems probable that the similar to—or identical with—lactic oxidase I. But the author cannot agree with

Edson, who maintained that enzyme in question oxidizes lactate to pyruvate in the presence of methylene blue. The authors consider that the enzyme preparation contains the two distinct lactic oxidases, characteristic properties of which are summarized in Table IX, and that among these lactic oxidase I is a new enzyme which catalyzes lactic acid oxidation.

TABLE IX

	Lactic oxidase I	Lactic oxidase II
The method ox extraction	Alternative freezing and thawing	Autolysis from acetone powder
Heated at 50° for 10 min.	Labile	Stable
Dialysis	Stable	Labile
Precipitation with acetic acid	Stable	Labile
Reaction with molecular oxygen	Directly	Requires the addition of carrier
Methylene blue or cytochrome c	No effect	Requires one of them as the carrier
Prosthetic group or cofactor	FAD	DPN
Reaction product	Acetate	Pyruvate

SUMMARY

1. Two distinct lactic oxidases were extracted from *Mycobacterium tuberculasis avium*.

2. One lactic oxidase was readily extrated by alternative freezing and thawing and partially purified by alternative precipitation with acetic and ammonium sulfate.

3. This enzyme reacted directly with molecular oxygen without the mediation of hydrogen carriers.

4. The Michaelis-Menten constant was approximately 5×10^{-2} *mole per liter*.

5. The enzyme was cyan-insensitive, but silver- and coppersensitive.

6. The enzyme was stable agaiast long-lating dialysis at 0° and ammonium sulfate precipitation. No easily dissociable cofactors was

demonstrated.

7. Since the contents of FAD in the enzyme preparation increased remarkably with the increasing degree of purification, it appeared that the prosthetic group of the enzyme was FAD.

8. One mole of oxygen was consumed and each 1 mole of carbon dioxide and volatile acid was formed respectively in the oxidation of 1 mole of lactic acid. Apparently neither pyruvic acid nor hydrogen peroxide was formed in the reaction. The catalyzed reaction can be presented as follows:

$$CH_3CHOHCOOH + O_2 \longrightarrow CH_3COOH + CO_2 + H_2O$$

This enzyme differs apparently from the lactic dehydrogenases of animal tissues, yeast, *E. coli*, *Gonococcus*, and higher plants. It is rather similar to the lactic oxidase of *M. phlei* reported by Edson.

9. The other lactic oxidase reacted with molecular oxygen by mediation of methylene blue, and was found to be unstable against short-lasting dialysis at 0°. The reaction catalyzed appeared to be as follows:

$$CH_3CHOHCOOH + \tfrac{1}{2}O_2 \longrightarrow CH_3COCOOH + H_2O$$

This enzyme was likely to be similar to the animal enzyme.

The authors wish to express a gratefull acknowledgment to Professor Saburo Watanabe, the chief of the Toneyama Institute for Tuberculosis, and Professor Siro Akabori of the Department of Chemistry, the Osaka University for their valuable criticisms throughout this investigation.

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SPECTROPHOTOMETRIC DETERMINATION OF BINDNING OF DYE BY PROTEINS

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Binding of dyes by proteins has been studied by many investigators using dialysis or spectrophotometric techniques. Klotz (*1*) and others used spectrophotometric techniques for these kinds of experiments and obtained results which were in general accordance with those obtained by dialysis experiments. In their studies, however, extinction coefficient of the bound dye was determined using dialysis equilibrium, in which concentrations of free dye inside and outside semipermeable membrane were thought to be equal, and extinction coefficient of the bound dye can, therefore, be determined by measuring relative transmission of the solution inside the membrane against the solution outside the membrane. Therefore, these kinds of spectrophotometric determinations depend on the dialysis oxperiments. In the present paper, the author attempts to determine the amount of bound dye without any aid from dialysis experiments.

Spectrophotometric Determination of the Bound Dye.

The intensities of lights traversed through dcm. thickness of protein, protein-dye mixture, and dye solutions, I_1, I_2, and I_3 are given by the following equations, Eq. (1), Eq. (2), and Eq. (3), respectively:

$$I_1 = I_0\ 10^{-EPd} \qquad \text{Eq. (1)}$$

$$I_2 = I_0\ 10^{-\{EP + kc\varepsilon_1 + (1-k)c\varepsilon_2\}d} \qquad \text{Eq. (2)}$$

$$I_3 = I_0\ 10^{-\epsilon_1 cd} \qquad \text{Eq. (3)}$$

where, I_0 is the intensity of the incident light, E, ε_1, and ε_2*, are molar extinction coefficients of protein, free dye, and bound dye respectively, and P, and c are concentrations of total protein and total

* Extinction coefficient of the bound dye is assumed to be independent of the total protein concentration. This assumption is valid if the protein concentration is sufficiently low, for example, less than 0.5 per cent for horse serum albumin solution.

dye, respectively, k is the fraction of free dye, *i.e.*, concentration of the free dye can be expressed as $k \times c$.

From Eq. (1), Eq. (2) and Eq. (3) we obtain,

$$E' = \log \frac{I_3}{I_1 I_2} = (\varepsilon_1 - \varepsilon_2)(1-k)cd \qquad \text{Eq. (4).}$$

As I_1, I_2, and I_3 are all measurable quantities, E' can be determined experimentally. Eq. (4) means that experimentally determined quantity, E', is proportional to the concentration of the bound dye, $(1-k)c$. Thus, if we can determine E', which is extinction coefficient of the bound dye, the absolute amount of the bound dye is readily obtained from Eq. (4).

Assuming that multiple absorption of dyes by protein follows the law of mass action, the following equation holds;

$$c_b = P \times f(c_f, p, q, \cdots) \qquad \text{Eq. (5).}$$

where c_f and c_b are the concentrations of free and bound dyes, respectively, and p, 1, $\cdots$ are parameters such as dissociation constants and maximum number of binding sites on protein molecule.

It is deduced from Eq. (5), that the concentration of the bound dye is proportional to the total protein concentration, if the concentration of free dye remains unchanged. Applying this relationship, we can determine the absolute amount of bound dye by the following procedure.

If we plot E' against total concentration, c, of the dye, for two concentrations of total protein, say, P and 2P, the resulting curve will be represented by

$$E_1'(c) = (\varepsilon_1 - \varepsilon_2)c_b d = (\varepsilon_1 - \varepsilon_2)dP \times f(c_f, p, q, \cdots) \qquad \text{Eq. (6),}$$

$$E_2'(c) = (\varepsilon_1 - \varepsilon_2)c_b d = 2(\varepsilon_1 - \varepsilon_2)dP \times f(c_f, p, q, \cdots) \qquad \text{Eq. (7),}$$

(*cf.*, Fig. 1). From these two equations, it can be proved that when E_1' and E_2' satisfy the relation,

$$2E_1'(c_1) = E_2'(c_2),$$

the concentrations of the free dye in both systems are equal, and the concentration of the bound dye in the second system (protein concentration 2P) is twice that of the first one (protein concentration P). Therefore, c_1 and c_2 must satisfy the following equations:

$$c_1 = c_d + c_f \qquad \text{Eq. (8),}$$

$$C_2 = 2c_b + c_f \qquad \text{Eq. (9).}$$

Subtracting Eq. (8) from Eq. (9), the concentration of the bound dye in the first system, c_b, can be obtained in the form;

$$c_b = c_1 - c_2 \qquad \text{Eq. (10),}$$

The concentrotion of free dye is evidently;

$$c_f = c_1 - c_b = c_2 - 2c_b \qquad \text{Eq. (11).}$$

Inserting the value of c_b thus obtained in Eq. (6) or Eq. (7), extinction coefficient of the bound dye is readily obtained. Fig. 1 shows the above mentioned procedure. This procedure can be applied to any system as long as the absorption equilibrium of the system is governed by the law of mass action.

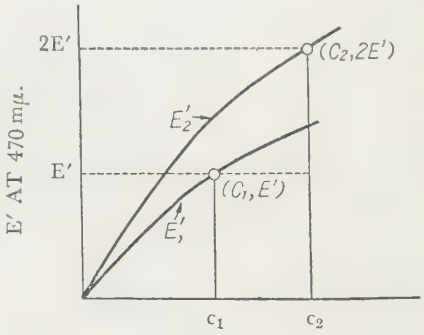

FIG. 1. Total concentration of methyle orange.

When absorption isotherms are expressed in a simple analytical form, concentration of the bound dye may be determined by more simple procedure. It was shown by many investigators (*1*) that for system containing methyl orange and serum albumin, the following relation holds;

$$C_b = pc_f(n/k)/1 + (c_f/k) \qquad \text{Eq. (12),}$$

where c_b, c_f, and P have the same meanings as before, n is the maximum number of binding sites on protein molecule, and K is the intrinsic equilibrium constant. Eq. (12) can easily be transformed to give;

$$\frac{P}{c_b} = \frac{K}{nc_f} + \frac{1}{n} \qquad \text{Eq. (13).}$$

As mentioned before, measurable quantity E′ is proportional to the concentration of the bound dye (Eq. (4)), Eq. (13) can also be written in the following form, if we designate the propotionality constant by κ;

$$\frac{P}{\kappa E'} = \frac{K}{n(c - \kappa E')} + \frac{1}{n} \qquad \text{Eq. (14).}$$

Now, if we measure E′ at three different values of total protein concentrations, say, P, αP, and βP, (total concentrations of the dye are the same in all three cases), three equations having the form analogous to Eq. (14) hold,

$$\frac{P}{n_\kappa E'} = \frac{K}{n(c - \kappa E')} + \frac{1}{n} \qquad \text{Eq. (15).}$$

$$\frac{\alpha P}{n_\kappa E'_\alpha} = \frac{K}{n(c - \kappa E')} + \frac{1}{n} \qquad \text{Eq. (16),}$$

$$\frac{\beta P}{n_\kappa E'_\beta} = \frac{K}{n(c - \kappa E')_\beta} + \frac{1}{n} \qquad \text{Eq. (17).}$$

Solving Eq. (15), Eq. (16), and Eq. (17) for n, κ, and K, we obtain;

$$\kappa=[E_{\alpha}(E'-E'_{\alpha})(E'_{\beta}-\beta E')-E'_{\beta}(E'-E'_{\beta})(E'_{\alpha}-\alpha E')]/E'E'_{\alpha}E'_{\beta}$$
$$\times[(1-\alpha)(E'_{\beta}-\beta E')(1-\beta)(E'_{\alpha}-\alpha E')]$$
$$K=(c-\kappa E')(c-E'_{\alpha})(\alpha E'-E'_{\alpha})/[E'_{\alpha}(c-\kappa E')-\alpha E'(c-\kappa E'_{\alpha})]$$
$$n=\kappa E'(K+c-\kappa E')/P(K-cE')$$

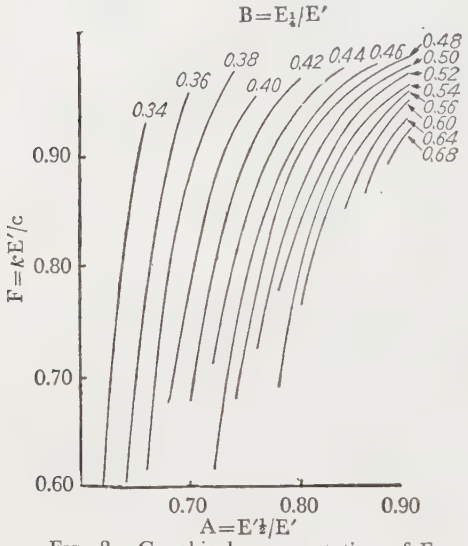

Fig. 2. Graphical representation of F.

It is convenient for practical purposes to put α and $\beta_{1/2}$ and $_{1/4}$, respectively. The author expressed the fraction of bound dye ($F=\kappa_c^{E'}$ at total protein concentration P as a function of $E'_{1/2}/F'=A$, and $E'_{1/4}/E'=B$, in the graphical form. (Fig. 2) Concentration of the bound dye can readily be obtained from the graph, if we measure E′, $E'_{1/2}$, and $E'_{1/4}$.

RESULTS AND DISCUSSION

Results obtained by the above mentioned procedures for the case of "methyl orange"—"horse serum albumin" saystem are shown in Table I. Detailed discussion of the results will be seen in the paper by

Table I

Material	pH	Buffer	$\varepsilon_2/\varepsilon_1$ (470 mμ)	n	Procedure*
Horse serum albumin	4.4	1/10 *M* acetate	0.42	~14	1
,,	6.7	1/30 *M* phosphate	0.56	~10	1
,,	6.7	1/10 *M* phosphate	0.60	~10	1
,,	7.8	1/30 *M* phosphate	0.56	~ 7	1
,,	11.2	1/30 *M* phosphate	0.63	~ 2	1
,,	6.7	1/30 *M* phosphate	0.54	~10	2

* 1; Procedure illustrated in Fig. 1.
2; Procedure using Fig. 2.

Kusunoki in this journal, and the author discusses here only on merits and drawbacks of the present procedure.

One of the merits is that the spectrophotometric measurements are simple and time consuming. Thus the hydrolysis or denaturation of proteins by contaminating sustances in the preparation can be avoided. Furthermore, measurements at different temperatures can be made very easily. Secondly, amounts of samples neccessary are small compared with those for dyalysis experiments.

On the other hand, the drawback of the procedure is that it depends on the spectrophotometric measurements, and the concentration of the dye must lie in the range where Lambert and Beer's law has its significance. This means that dye concentrations are restricted to the small range only. In the case of methyl orange, the concentration of the dye must be below $1\times10^{-4}\,M$. Furthermore, ε_1 and ε_2 must be so different from each other as to be able to be determined with sufficient accuracy. In most cases difference between ε_1 and ε_2 are relatively large and this drawback can be a serious one, if measurements are carried out with monochromatic or nearly monochromatic ligts (band width less than 10 mμ).

The first procedure enables us to determine the amount of bound dye for general system of absorption equilibrium, therefore, competitive absorption or inhibition of absorption by other substances must be carried out by the first procedure. On the other hand, second one is convenient for simpler cases of absorption equilibrium, such as "serum albumin"-"methyl orange" system.

The author expresses his thanks to Prof. K. Kodama and members of his laboratory for their kindness during these investigations.

SUMMARY

New procedures for the determination of the amount of bound dye have been devised and their merits as well as drawbacks are discussed. Some of the results obtained by these procedures are reported.

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A STUDY ON THE BINDING OF DYES BY PROTEINS

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Playing many kinds of biological roles, plasma proteins combined with various substances of biological importance are circulating steadily in animal bodies. Many attempts have been made recently to clarify the relation between biological functions and physicochemical properties of plasma proteins. (*1*) The author attempted to determine quantitatively the binding of dyes of known structure by proteins from the point of view that such a determination may enable us to obtain some information on the inter- and intra-molecular configuration of protein molecules.

In 1946 Klotz *et al.* (*2, 3*) reported for the first time that binding of several sorts of azo dyes by bovine serum albumin followed the simple law of mass action and that the determination of the amount of bound dye could be carried out with the aid of equilibrium dialysis method alone or together with spectrophotometric technique. However, it is very difficult to determine the maximum number, denoted by n-value throughout this article, of dyes by proteins. Furthermore, the choice of dialysis membrane is a difficult problem, because in most cases the adsorption of dyes on membrane itself disturbs the results obtained by dialysis experiments.

Lately a new spectrophotometric determination of the amount of bound dye by proteins was devised on our laboratory and published in this journal (*4*). The new procedure depends only on spectrophotometric measurements and is free of the complications arising from dialysis process. In the present paper the results obtained by the new procedure on the binding of dyes by native and denatured proteins will be reproted together with the discussion on the difference in configuration of various kinds of proteins based on the experimental results.

EXPERIMENTAL

Proteins used in the present experiments were horse serum albumin, human serum albumin, horse gamma-globulin ahd clupein sulfate. The

dyestuffs emyloyed were methyl orange, phenol red, and congo red as acidic dyes and methylene blue, toluidine blue, and neutral red as basic ones.

The Methods of Preparation of Native Proteins:

Human and Horse Serum Albumin—Serum albumin was crystallized two times following Kekwick's method (*5*). In some cases amorphous preparations without crystallization were employed.

Horse Serum Gamma-Globulin—Gamma-globulin was isolated by salting out with Na_2SO_4, followed by dialysis against 0.9 per cent NaCl.

*Clupein Sulfate**

Purities of all three protein preparations were above 90 per cent electrophoretically. Protein concentrations were determined by N determination with micro-Kjeldahl method.

Dyestuffs—Each dyestuff was obtained from Merck or Kahlbaum Company with or without recrystallization.

Spectrophotometry—All experiments were carried out by spectrophotometric technique, principle and practice of which is described in the paper by Shimao (*4*). The main procedure consists in measuring E′(*cf.*, Eq. (4) of the paper by Shimao (*4*) at the wave length where free dye shows its maximum absorption. Total concentration of the dye was such that the absorption of light followed Lambert and Beer's law where the state of the dissolved dye was thought to be monodisperse.

pH-Measurements—pH of the solution was determined by antimony electrode in all cases.

RESULTS

Binding of Acidic Dyes by Native Proteins—Results of the quantitative determinations of the bidning of acidic dyes by native proteins of serveral kinds are summarized in Table I.

While serum albumin shows high binding ability, horse serum gamma-globulin seems to combine neither with methyl orange nor with phenol red, for the dyes show no shift of extinction in the presence of gamma-globulin. When 0.5 per cent solution of gamma-glabulin was mixed with $5.00\times10^{-5}\,M$ solution of congo red in the medium more acidic than pH 6.7, which is the isoelectuic point of the protein, turbity or flocculate appeared immediately and apparently, although both gamma-globulin and congo red solutions remained clear at the same pH. When

* Culpein sulfate was kindly given by Prof. Ando of Institute of Science and Technology of Tokyo University.

TABLE I

Characteristics of Binding of Dyes by Proteins

Protein	Dye	pH	Temperature	$k \times 10^5$	$k_1 (=n/k) \times 10^{-5}$	$\triangle F_1$	n
						cal./mole	
Horse serum albumin (crystallized)	M.O.*	6.8	28°	2.22	3.60	−7800	8.0
Horse serum albumin (isoelectric precipitation)	,,	,,	,,	2.88	3.00	−7500	8.6
Horse serum albumin (in horse plasma)	,,	,,	27°	2.82	3.05	−7500	8.6
Human serum albumin	,,	,,	20°	1.54	3.90	−7480	6.0
Clupein sulfate	,,	,,	27°	0.77	13.40	−8400	10.4
Horse serum albumin	Ph. R**	7.8	27°	1.08	3.70	−7800	4.5

* Methyl orange. ** Phenol red. k : Intrinsic dissocaition constant.
k_1 : First association constant. $\triangle F_1 : -RT\,ln k_1$.

centrifuged for 15 minutes, these flocculates sedimented easily, and the concentration of the dye in supernate was found to be decreased showing that the flocculates were complexes of dye and protein.

Ih order to obtain information as to the mechanism and binding sites of protein for dyes in more detail, the following experiments were carried out on the binding of methyl orange by horse serum albumin.

Effect of Temperature—Fig. 1 shows the comparison of effects of various temperatures on the amount of bound dye at pH 6.8. As shown in the

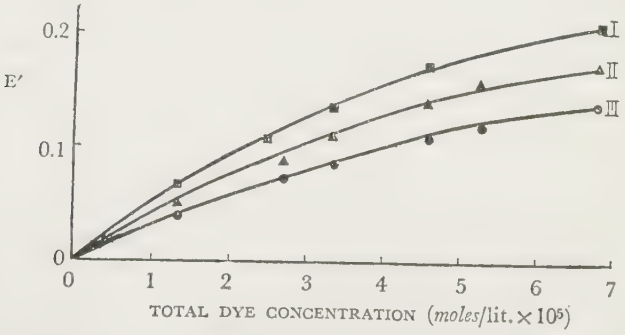


FIG. 1. Effect of temperature on binding of methyl orange by horse serum albumin at pH 6.8.
I : 15% ; II : 23° ; III : 28° ; Concentration of albumin : 0.2%.

figure, the lower the temperature, the more the amounts of dye combined with protein. Binding of the dye by protein was found to be completely reversible as long as the protein remained native.

Effect of pH—As shown in Fig. 2, the shift of the pH of the medium to acid side is accompanied by an increase in the amount of bound dye, while in alkaline medium the amount of bound dye decreases. No significant difference could be observed caused by the difference in the kind of buffer ions used.

In addition to the experiments described above, measurements of

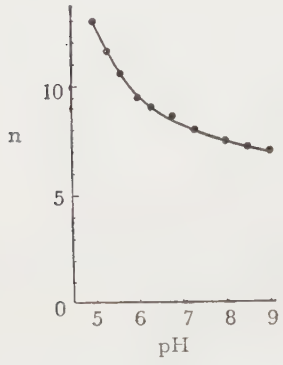


FIG. 2. Effect of pH on binding of methyl orange by horse serum albumin. Concentration of albumin; 0.2%

Concentration of methyl orange: 7.0×10^{-5} *M*; temperature, 26°.

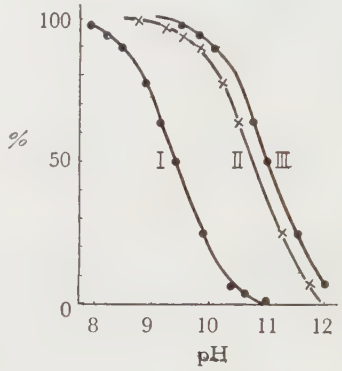


FIG. 3. Comparison of the curve of n-value and dissociation curves of ε-ammonium and guanidinium group in protein.

I: Dissociation curve of ε-ammonium group. II; Curve repredenting n-value for binding of methyl orange by horse serum albumin (per cent of n-value at pH 8.5). III: Dissociation curve of guanicinium group.

n-values were carried out in the medium, pH of which lay near the pK's of ε-ammonium and guanidinium groups of proteins. These were perfomed to compare dissociation curves of the two groups with the change in n-value with pH of the medium. Results are shown in Fig. 3. Curve representing n-values falls between dissociation curves of ε-ammonium and guanidinium groups in protein molecules as shown in the figure. At pH 10.75 n-value becomes one-half the value at pH 8.5, while 50 per cent

dissociation of ε-ammonium and guanidinium groups in proteins occurrs at pH 9.4 and 11.0, respectively.

Binding of Methyl Orange with Clupein Sulfate—As shown in Table I, methyl orange combines with clupein sulfate in the absence of phosphate buffer. Over 87 per cent of amino acid residues of clupein sulfate are arginines, and the total number of basic groups per molecule becomes 20, if we assume the molecular weight of clupein to be 5000. The ratio

$$\frac{\text{n-Value for methyl orange}}{\text{Total basic groups per protein molecule}}$$

is 0.5 for clupein sulfate, while this ratio is 0.08 for horse serum albumin.

Binding of Basic Dyes with Native Proteins—With methylene blue, toluidine blue, and neutral red, no shift of extinction was observed regardless of the kinds of proteins, pH of the medium, and concentrations of both dye and proteins. It will be sure, therefore, that there is no interaction between these kinds of dyes and proteins causing complex formation.

Binding of Methyl Orange with Denatured Serum Albumin—From several experimental results described above, it would be certain that the binding of dyestuff by proteins is affected by numerous complicated factors; they are, for example, internal structure, surface configuratirn of proteins and arrangements of amino acids composing protein molecules. If, therefore, the configuration of protein molecules deviates from native one after denaturation, the amount of bound dye will be altered and this alteration will be regarded as a good measure of degree of denaturation. From this view point, the following experiments were carried out to investigate the binding between methyl orange and denatured horse serum albumin.

Acid- and Alkaline-Denaturation—To 0.2 per cent solution of horse serum albumin, 1 *N* H_2SO_4 or NaOH aqueous solution was added to the final concentration of 0.3 *N* and pH was brought back to 6.8 again by neutralization with NaOH or H_2SO_4, respectively. Comparison of the amount of bound dye of thus treated protein with native one is shown in Fig. 4. In the case of acid treated albumin decrease in the amount of bound dye was only a slight one, while rapid and considerable decrease was observed in the case of alkaline treated one.

Heat Denaturation—A 0.2 per cent aqueous solution of protein was heated at the temperature of 45°, 50°, 55°, 60° and 80°. After being cooled in cold water rapidly, the amount of bound dye was measured. The results are shown in Fig. 5. The figure indicates that protein shows a tendency to lose its ability of dye binding when heated at temperature above 50°, for

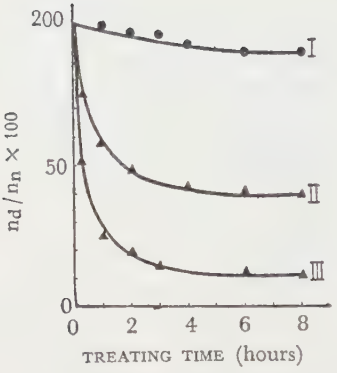


Fig. 4. Effect of acid or alkali treatment of horse serum albumin on n-value.
I: 0.3 N H_2SO_4, 37° II: 0.3 N NaOH, 15° III: 0.3 N NaOH, 37°. Concentration of albumin: 0.2%, Concentration of m.o.: 5.00×10^{-5} *M*.

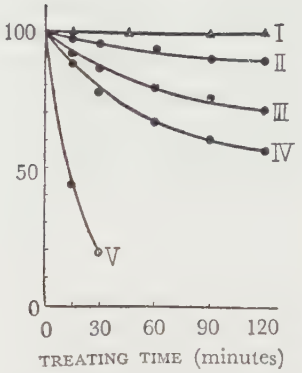
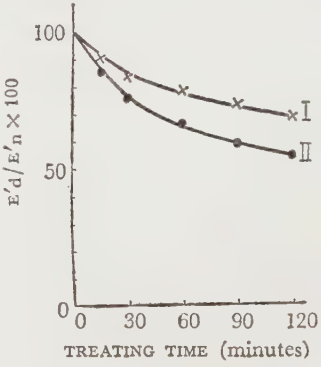


Fig. 5. Effect of heat treatment of horse serum albumin on its methyl orange binding ability.
I: 45°. II: 50°. III: 55°. IV: 60°. V: 80°. Concentration of albumin: 0.2%. Concentration of m.o.: 5.00×10^{-5} *M*. pH 6.8.

Fig. 6. Effect of previous treatment with acid or alkali on binding of methyl orange by heat treated horse serum albumin.
I: Without previous treatment or previously acid treated. II: Previously alkali treated. Concentration of albumin: 0.2%. Concentration of m.o.: 5.00×10^{-5} *M*. pH 6.8



one hour. The higher was the temperature, the more remorkable was this tendency. As shown in Fig. 6 protein previously treated with acid shows decrease in its ability of dye binding at the same rate as native protein, protein previously treated with alkali, on the contrary, loses its ability at far faster rate than native one.

Renaturation of Protein—The Protein which has lost its dye binding ability by heating at 60° for 30 minutes regains this property by standing at lower temperature; this recovery is, however, incomplete. These circumstances are illustrated in Table II. Rapidly cooled protein recovers its dye binding ability more slowly than the gradually cooled one. In addition, rapid and higher extent of recovery was observed when denatured protein was cooled in the presence of the dye as shown in Table III. Results of experiments on denatured proteins described above are not altered by the presence or absence of phosphate buffer ions.

TABLE II

Renaturation of Heat Treated Horse Serum Albumin in the Absence of Dye
(Results are shown in per cent of the native protein)

Time after cooling	Material	
	Rapidly cooled	Gradually cooled
hours	*per cent*	*per cent*
1	79.0	84.0
2	81.5	88.2
4	85.1	89.3
8	90.2	92.8
24	94.0	94.1

TABLE III

Renuration of Heat Treated Horse Serum Albumin in the Presence of Dye
(Results are shown in per cent of the native protein)

Time after cooling	Material	
	Rapidly cooled	Gradually cooled
hours	*per cent*	*per cent*
1	85.8	89.7
2	90.3	92.7
4	95.3	97.2
24	98.3	98.4

DISCUSSION

It is clear from the above experiments that the new spectrophotometric procedure enables us to make the acquaintance of the amount of dyes

bound by proteins very conveniently, and with toe aid of this procedure, one can catch even a slight change in binding ability of proteins.

With regard to n-value, the results obtained in these experiments, *i.e.*, 8—9 moles methyl orange per mole of horse serum albumin are considerably different from the one obtained formerly by Klotz *et al.*, *i.e.*, 22.3 moles methyl orange per mole of bovine serum albumin (*2*). This discrepancy may be due, besides to the difference in the method of determination and the source of material, mainly to the molar concentration of dye solutions used, that is, Klotz *et al.* used a solution of methyl orange whose concentration lay above 1×10^{-4} *M* where the dye particles in solution were thought to be not monodisperse, because in this range of concentration, optical densities of the solution do not follow Lambert and Beer's law.

For a long time it has been recognized that albumin in blood plasma combines reversibly with various kinds of organic and inorganic substances such as drugs, lipids or carbohydrates, *etc.*, in soluble state. (*7*) Results obtained in this experiment that albumin, not only in pure solution but also in plasma, specifically combines with acidic dyestuffs agree with the above mentioned concept and seem to be of biologicaly interest. With regard to the sites of interaction between bovine serum albumin and acidic azo dyes whose SO_3^- groups dissociate completely under experimental conditions, Klotz and Teresi (*8*, *9*), suggested that ε-ammonium groups or guanidinium groups were responsible referring to their experiments on acetylated, formolated or guanidinated proteins.

The gradual increase in the binding number of dye when pH of the medium are changed from 9 to 5, suggests that histidine imidazole groups take part for the binding of dyes. ε-ammonium and guanidinium groups are also thought to be the sites of dye binding on protein molecules, since, as shown in Fig. 3, n-value decreases in the range of pH where dissociations of these groups are suppressed. Further consideration on the magnitude of ΔF_1 and its temperature dependence leads us to the inference that the force acting between protein and dye molecules is of van der Waals type. The fact that serum albumin and serum gamma-globlin, although they have similar values of the

$$\frac{\text{Total number of free basic groups}}{\text{Total number of free acidic groups}}$$

ratio, are essentially different in the binding ability suggests that factors other than net charge of the protein, such as surface configuration of

dyes and proteins and arrangements of amino acids in protein molecules, also influence the binding ability markedly.

Therefore, it may be naturally expected that the change in the amount of bound dyes would occur, if by means of denaturing agents, a partial or total rupture of configuration is induced in the protein molecule. Conversely, if we determine the change in the amount of dyes bound, degree of denaturation can be known. The fact that serum albumin shows considerable decrease in the dye binding ability when heated at 50 to 55° indicates that spectrophotometric measuremment is excellent method for the detection of denaturation of proteins.

The highest degree of decrease in the amount of bound dye caused by alkaline treatment, shown in Figs. 4 and 5, might be explained by the aggregation of albumin molecule caused by alkali, which was previoulsy discovered by Heidelberger (*10*) in his studies on denaturation of egg albumin. Alkaline hydrolysis of guanidinium group of arginine might also take part in the phenomenon. As mentioned before, acid treated serum albumin showed only a slight decrease in binding ability when measured in neutral solution. Although we have no information on the dye binding ability of acid denatured protein itself because of the fact that change in the state of dissociation of methyl orange occurrs between pH 4 and 5, and the mode of interaction between protein and dye changes considerably, the above mentioned slight decrease in binding ability is probably due to the recovery of the ability when denatured proteins are brought back to neutral pH.

Decrease in the amount of bound dye accompanying denaturation can be interpreted in the following ways; (a) Salt linkages or hydrogen bonds in native protein molecules are split by denoturing agents, and they form new kinds of bonding when denaturing agents are taken away from the system, and potein molecule takes new configuration more or less unsuitable for binding of dyes; (b) As a result of denaturation, protein loses its whole structure and free basic and acidic groups approach each other to form new inter-or intra-molecular linkage in the denatured state. Because of the bond formation between free basic groups and other polar groups, the number of binding sites is reduced, and the decrease in binding ability occurrs as a consequence.

Results represented in Fig. 6 mean that although alkali denaturation recovers its ability of dye binding by neutralization, ability thus recovered is more easily destroyed by heat treatment compared with native and acid treated proteins.

Recovery of heat treated albumin is, as shown in Tables II and III,

dependent on cooling process. The higher degree in the recovery of binding ability of heat treated protein observed in the presence of dye anions indicates that denatured disoriented protein molecule reorients itself so as to take configuration complementary to the substance which coexists. We can understand the importance of steric configurations of acidic and basic groups and other polar groups in portein molecule from these considerations.

SUMMARY

The nature of the binding of acidic and basic dyes with native and denatured plasma plasma proteins and clupein sulfate was studies spectrophotometircally. Results obtained at different pH, and temperatures, for native and denatured proteins were discussed from the standpoint of protein structure.

The author thanks Prof. K. Kodama, Dr. H. Yoshikawa, H. Hirai, and Mr. K. Shimao for their valuable kindness throughout these investigation.

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THE METABOLISM OF DEHYDROCHOLIC ACID IN ORMANISM

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(Received for publication, December 24, 1951)

T. Fukui (*1*), T. Mori (*2*), and TaiSihk Sihn (*3*), have shown that, in the culture medium of *Bacillus coli communis*, dehydrocholic acid is reduced to 7-oxy-3, 12-diketocholanic acid, and dehydrochenodesoxycholic acid to chenodesoxy-cnolic acid. It has been reproted that by the action of yeast (*3*), only the ketone radical of the C_3 position of dehydrocholic acid is reduced to reducto-dehydrocholic acid, whereas the C_{12}-ketone radical of the bile acid nucleus does not undergo such reduction. But when the C_{12}-ketonic bile acids were administered to animals, it was reduced and excreted as hydoxy compounds, as clearified by Chai Heung Kim (*5*), N. Takemoto (*6*), and Y. Arata (*7*).

The author has investigated the posibility that the C_3-ketone radical is reduced in liver and that the C_7 and C_{12}-ketone radicals are reduced in another organ (*8*). In this paper another evidence will be presented for the reduction of dehydrocholic acid to cholic acid under the action of some micro-organisms.

EXPERIMENTAL

Into 2 liters of 1.25 per cent sodium dehydrocholate solution, well minced fresh pancreas out of four bovines was thrown and the whole solution was incubated at 37°. Ten days later, two fresh pancreases were again added to the solution, and this mixture was left standing to putrefy for 10 days. On examining the bacteria in the putrefied fluid, there were observed *Staphylococci* and *Pneumococci*, but not *Enterococci*. This blackish-brown decayed fluid was then concentrated and dehydrated. Then the material was extracted with alcohol, from which alchol was removed. After removal of the fatry substance with petroleum ether, it was made alkaline with a 5 per cent sodium carbonate solution, filtered, and then acidulated with dilute hydrochloric acid. The precipitate was dehydrated and extrated with ether. The ether solution was then evaporated and a reddish-brown muddy residue was obtained.

This residue was dissolved in 5 per cent sodium hydroxide solution and after hycrolysis for 1 hour at room temperature, the hydrolyzate was extracted with petroleum ether, while being acidified with hydrochloric acid. The remaining precipitate was extracted with ether and separated into two parts: one ether-soluble (B) and another ether-insoluble (A).

The ether-insoluble portion (A) did not crystallize from acetone, and so it was dissolved in 2 per cent ammonium solution, to which 10 per cent barium chloride solution was added. The barium salt was separated into two parts: one water-soluble and another water-insoluble. When the wate-soluble barium salt solution was concentrated to one-third of its volume, the colored precipitate was separated, and, was recrystallized from ethylacetate and alcohol under usual treatments, yielding 18 mg. of cholic acid (m.p., 198°). The mother liquor of barium salt solution was again concentrated to one-third of its volume and 42 mg. of cholic acid were similary obtained from the separated precipitate. Of the remainder, the most water-soluble barium salt portion was converted to a free acid by the usual method. On recrystallizing from ethyl-acetate, a crystalline precipitate was separated, from which 193 mg. of cholic acid (m.p., 198°) were gaiaed. When the mother liquor of ethyl-acetate solution from which cholic acid had been removed, was suitably concentrated and left standing, bright plate-like crystal (m.p., 213°—215°) separated out, and on recrystallizing from alcohol, 6 mg. of bright plate-like crystals (m.p., 218°) were obtained. In the same way, from the mother liquors, which had the crystals previously removed, 10 mg. of crystals (m.p., 218°) were obtained. These crystals showed neither Hammarsten's nor Jaffe's reaction. The mel ing point was lowered when mixed with pure 3,7-dioxy-12-ketocholanic acid (m.p., 220°). This acid was likely to be entriely different from the above acid and, although there has been known no bile acid with similor analytical properties, the cleavage in the side-chain might be expected to occur.

Anlayses: Substance. 2.850 mg.; CO_2, 7.31 mg.; H_2O, 2.27 mg.;
$C_{22}H_{34}O_5$: Calcd. C 69.84%; H 8.99%
Found. C 70.00%; H 8.91%

The ether-soluble potion (B) was dissolved in 2 per cent ammonium solution to which 10 per cent barium choloride solution was added, and was separated into water-insoluble and water-soluble barium salts. On slightly concentrating the solution of water-soluble barium salt, the brown jelly-like precipitate was separated, and re-crystallized as a free acid from ethyl-acetate by the usual method. There were obtained

35 mg. of cholic acid (m.p., 198°). The mother liquor was further concentrated to one-third of its volume, and then from the separated barium salt 15 mg. of cholic acid were obtained. The original solution containing the easily dissolved barium salt was further concentrated to four-fifths of its volume and a large amount of brown jelly-like barium salt was separated. The precipitate was treated with sodium carbonate, then re-crystallized as a free acid from ethyl-acetate and then from alcohol, successively. This recrystallization was repeated and from this fraction, a total of 101 mg. of cholic acid (m.p., 198°) was obtained. The most easily dissolved barium salt solution was immediately acidulated with dilute hydrochloric acid. The formed precipitate was washed with water, dried, and plate-like crystal (m.p., 213°) was obtained after recrystallizing from ethyl-acetate and then with alcohol, successively. This crystal was further recrystallized from alcohol-water, yielding 9 mg. of plate-like crystals (m.p., 218°). This acid was identified with that obtained as a fraction of (A). Even though the original solution from which all crystals had been removed, was purified, no trace of dehydrocholic acid could be detected, despite that there were 3 g. of non-crystalline oil.

Cholic acid analyses:

Substance, 3.102 mg.; CO_2, 7.803 mg.; H_2O 2.699 mg.;

$C_{24}H_{40}O_5 \cdot C_2H_5OH$ Calcd. C 68.72%; H 10.13%

Found. C 68.65%; H 9.74%

CONCLUSION

The bacteria concerned in this experiment were *Staphylococci* and *Pneumococci*, which reduced not only the ketone radicals of C_3 and C_7, but also that of C_{12} of the bile acid nucleus of dehydrocholic acid, and consequently produced cholic acid. Furthermore, the fact that each ketone radical is mainly reduced to α-type of alcohol radical, may have an important significance in the acceptance of an alcohol radical by the C_{12} during the process of bile acid formation in the body. Also at present, a very thorough investigation is being continued to seaach which organ has the highest possibility of effecting this change.

Due to the small quantity of bile acid obtained here (m.p., 218°), the elucidation of its structure must await future experiments, but the results of analyses may suggest the occurrence of a cleavage in the side-chain of 3,7-dioxy-12-keto-bisnorcholanic acid, in which the alcohol radicals of C_3 and C_7 are reduced (*9*).

The author wishes to express his sincere appreciation to Profs. T. Shimizu and T. Kazuno for their encouragement and advices during the course of this work. Present research has been defrayed by the aid of the Science Research Fund of the Ministry of Education.

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The Journal of Biochemistry, Vol. 39, No. 3.

STUDIES ON THE MECHANISM OF ACTION OF CHOLINESTERASE

I*. KINETIC STUDIES ON THE ACTION OF HYDROGEN IONS UPON CHOLINESTERASE

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(Received for publication, January 16, 1952)

The properties of cholinesterase have already been the subject of repeated study in the field of enzymology as well as physiology, but relatively little information is avaiable concerning the physico-chemical nature of the enzyme and the mechanism involved in its action. The present series of the study was begun with the view to filling up this blank, using prinicipally kinetical methods in our approach to the problem.

In the present paper are described the experiments dealing mainly with the effect of hydrogen ions upon the enzyme. Evidence will be presented showing that the substate (acetylcholine chloride) and hydrogen ion compete with each other for the molecule of enzyme. By the analysis of the data obtained it was concluded that the hydrogen ion attacks a certain amino group in the enzyme molecule which is essential for its catalytic action.

EXPERIMENTAL METHOD

Throughout the experiments in this work equine serum was used as the enzyme and acetylcholine chloride as the substrate. The hydrolysis of the substrate was followed by potentiometric titration using a glass electrode (*1*). The equine serum (0.2~0.3 ml.) was mixed with 20 ml. of distilled water in a reaction vessel placed in a thermostat and the pH of the mixture was regulated to a desired value by adding sodium hydroxid or hydrochloric acid. After leaving the mixture for about 15 minutes so that the equilibrium between enzyme and the hydrogen

* The first report on the present series of work was made at the Symposium on Enzyme Chemistry held by the Chemical Society of Japan in Tokyo, in October, 1950.

ion had been attained, a certain amount of acetylcholine chloride solution was added, and keeping the pH value of the mixture constant, the liberated acid was titrated with sodium hydroxide (0.02 *N*). The titration was continued for about 15 minutes and taking the initial linear part of the titration curve, the rate of hydrolysis was determined. Parallel with each experiment a control test was run without the addition of enzyme, and the rate of enzymatic action was determined taking into consideration the rate of the non-enzymatic hydrolysis.

RESULTS

I. *Relationship between the Rate of the Enzymatic Hydrolysis and the Hydrogen Ion Concentration*:

The effect of hydrogen ions upon the activity of the enzyme was found to be a function of temperature and the concentration of the substrate added. As an example, the result obtained at the substrate concentration of $10^{-2.4}$ mole/lit. and at 33° is given in Fig. 1, from which

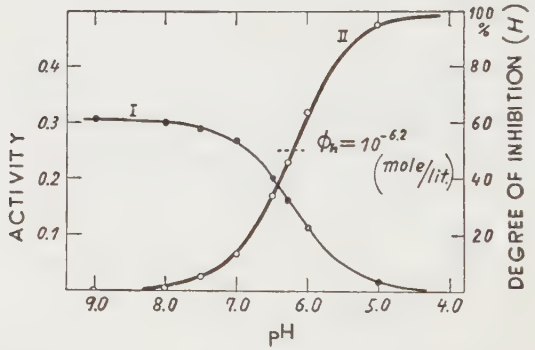

Fig. 1. "Activity-pH" relationship (I) and the relationship between the degree of inhibition and pH (II).
Substrate concentration: $10^{-2.4}$ mole/lit.; Temperature: 33°.

it may be seen that an increasing inhibition took place in the acid region from pH 7 towards pH 5.

Regarding the activity at pH 9.0 as the maximum (non-inhibited) value, we may define the degree of inhibition (H) caused by higher hydrogen ion concentration as follows:

$$H = \frac{v_0 - v}{v_0} \qquad \text{Eq. (1)}$$

where v_0 is the maximum rate and v the reduced rate observed at lower pH values. Curve II in the figure represents the "inhibition-pH-curve" obtained by plotting the value H against pH. This curve was found to be represented by the equation:

$$H=\frac{[H^+]}{[H^+]+\phi_h} \qquad \text{Eq. (2)}$$

where ϕ_h is a constant corresponding to the hydrogen ion concentration causing 50 per cent innibition. Experiments under various conditions showed that by and large the relationship expressed by Eq. (2) holds true, though in some cases, some apparent deviation from this equation were observed. This may be attributted to the situation that the "maximum" rate at higher pH values did not represent the real non-inhibited rate of the reaction. In these cases it was found that the reciprocals of the reaction rates observed at lower pH values relate linearly to the hydrogen ion concentration. By extrapolating the straight line to $[H^+]\rightarrow 0$, we obtain the value of rate which may be regarded as being the real value of v_0. If this value was applied in evaluating H according to Eq. (1), a satisfactory fit of Eq. (2) was found also in these cases.

Experiments carried out under various experimental conditions showed that the value ϕ_h in Eq. (2) is a function of the substrate concentration and of temperature. In the following experiments the modifications of ϕ_h brought about by these experimental conditions were investigated in some detail.

Effect of the Change in Substrate Concentration [S] *upon* ϕ_h *at* 33°—The effect of substrate concentration upon the value of ϕ_h was investigated at the temperature of 33°. When ϕ_h was plotted against [S], a straight line was obtained as shown in Fig. 2.

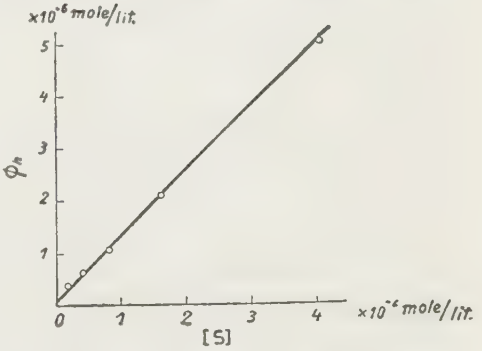

FIG. 2. Relationship between ϕ_h and the substrate concentration [S] (at 33°).

Effect of Temperature upon ϕ_h *at a Sufficiently High Concentration of the Substrate*—The values of ϕ_h were determined at different temperatures (13°-40°) using the substrate in concentration of $10^{-1.6}$ mole/lit. which has been confirmed to be saturating for the enzyme

action. A plot of the logarithm of ϕ_h thus obtained against the reciprocal of absolute temperature gave a straight line as shown in Fig. 3.

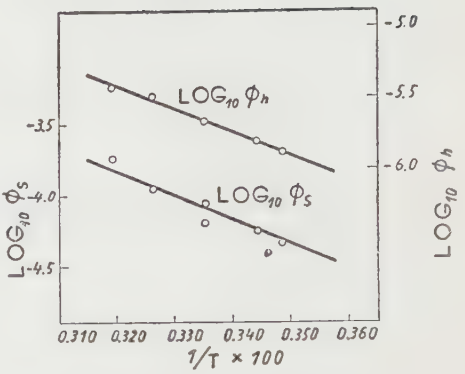

Fig. 3. Effect of temperature upon ϕ_h and ϕ_s.

II. *Relationship between the Rate of Enzymatic Hydrolysis and the substrate Concentration*:

It has already been demonstrated by earlier investigators (*1*, *2*) that the activity-pS-relationship (pS: the logarithm of reciprocals of the substrate concentration) of serum cholinesterase can be represented by an S-shaped curve which corresponds to the equation:

$$v=\frac{[S]V_{max}}{[S]+\phi_s} \qquad \text{Eq. (3)}$$

where ϕ_s is the Michaelis' constant and V_{max} a constant corresponding to the maximum reaction rate to be observed at a sufficiently high concentration of substrate.

Applicability of this equation under various experimental conditions was ascertained also in our experiments.* Investigations were, therefore, made to determine whether and in what manner the value ϕ_s is modified by changes in pH and temperature.

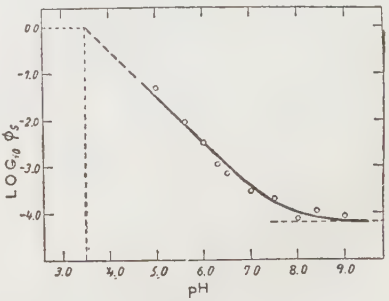

Fig. 4. Relationship between ϕ_s and pH (at 33°).
(○): Observed value. (----): Calculated according to Eq. (12). ($K_m=10^{-4.2}$ mole/lit., $K_h=10^{-7.7}$ mole/lit.).

Effect of the Change in Hydrogen ion Concentration upon the Michaelis' Constant—A series of experiments carried out in the pH range between 5.0 and 9.0 and at a constant temperature 33° gave the results which are summarized in Fig. 4. As may be seen

* In some cases the value V_{max} in Eq. (3) could not be obtained directly by experiment. In these cases it was determined by plotting $1/v$ against $1/[S]$ and by extrapolating the straight line obtained to $1/[S]\rightarrow 0$.

from the figure, the value of $\log_{10}\phi_s$ plotted against pH decreases linearly in the acid region of pH and in alkaline region it approaches asymptotically to a definite value—4.2. Noteworthy is the fact that in the acid region the linear portion of the curve shows the tangent of -1.

Effect of Temperature upon the Michaelis' Constant—In view of the fact that the Michaelis' constant showed a definite constant value in the sufficiently alkaline range of pH, the experiments under different temperatures (13-40°) were carried out at pH 8.4. The results obtained are illustrated in Fig. 3, where the logarithms of ϕ_s are plotted against the reciprocals of absolute temperature. As may be seen from the figure, an approximately linear relationship exists between these two values.

DISCUSSION

In interpreting the various facts we have observed, we may picture the mechanism of the action of cholinesterase as follows:

$$E + S \underset{k_2}{\overset{k_1}{\leftrightarrows}} ES \qquad \text{Eq. (4)}$$

$$ES \underset{k_3}{\rightarrow} E + P \qquad \text{Eq. (5)}$$

where E is the free enzyme, S the substrate*, ES the enzyme-substrate-complex and P the reaction products. To account for the effect of hydrogen ion upon the enzyme action it may be reasonable to assume that, besides the reactions mentioned above, there occurs a reaction between E and H^+ forming an inactive molecule EH^+ in the manner:

$$E + H^+ \underset{K_h}{\rightleftarrows} EH^+ \qquad \text{Eq. (6)}$$

The equilibrium constant of this reaction may be denoted by K_h. On the assumption that S and H^+ compete with each other for E, the stationary rate of hydrolysis is given by

$$v = \frac{k_3 \varepsilon}{1 + \frac{K_m}{[S]}\left(1 + \frac{[H^+]}{K_h}\right)} \qquad \text{Eq. (7)}$$

where ε denotes the total concentration of the enzyme and K_m the Michaelis' constant $(=k_2+k_3)/k_1$. If the hydrogen ion concentration

* It should be remarked that in the whole pH range employed in our experiments, the molecules of acetylcholine chloride should be totally in ionic form.

is sufficiently low, so that $[H^+]$ is negligibly small compared with K_h, the rate of hydrolysis will be

$$v_0 = \frac{k_3 \varepsilon [S]}{[S] + K_m} \qquad \text{Eq. (8)}$$

Substituting the value v_0 and v in Eq. (1), we have

$$H = \frac{[H^+]}{[H^+] + K_h\left(1 + \frac{[S]}{K_m}\right)} \qquad \text{Eq. (9)}$$

Comparison of this equation with Eq. (2) leads to the relation

$$\phi_h = K_h\left(1 + \frac{[S]}{K_m}\right) \qquad \text{Eq. (10)}$$

This equation corresponds satisfactorily to the experimental result that the value ϕ_h was a linear function of [S].

From Eq. (7) it also follows that the maximum reaction rate to be observed at a sufficiently high substrate concentration will be

$$V_{max} = k_3 \varepsilon .$$

Substituting this relationship into Eq. (7) we have

$$v = \frac{V_{max}}{1 + \frac{K_m}{[S]}\left(1 + \frac{[H^+]}{K_h}\right)} \qquad \text{Eq. (11)}$$

which coincides well with Eq. (3) already established experimentally. The value ϕ_s, therefore, has the following implication:

$$\phi_s = K_m\left(1 + \frac{[H^+]}{K_h}\right) \qquad \text{Eq. (12)}$$

This deduction agrees with the observation that at higher hydrogen ion concentration a linear relationship exists between $\log_{10} \phi_s$ and pH with a tangent of unity, while at lower hydrogen ion concentration ϕ_s becomes independent of pH. Applying Eq. (12) to the data given in Fig. 4, we obtain the following figures:

$$\left.\begin{aligned} K_m &= 10^{-4.2} \text{ (mole/lit.)} \\ K_h &= 10^{-7.7} \text{ (mole/lit.)} \end{aligned}\right\} \text{ at } 33^\circ .$$

Approximately the same values for these constants are obtained by applying Eq. (10) to the data given in Fig. 2.

In our experiment the effect of temperature upon ϕ_h was measured under the condition of saturating substrate concentration. On the other hand the corresponding measurement was made for ϕ_s under the condition of sufficiently low hydrogen ion concentration. Taking these condition into account, we may derive from Eq. (10) and Eq. (12) the the following equations:

$$\frac{d \log_{10} \phi_h}{d(1/T)} = \frac{d \log_{10}(K_h/K_m)}{d(1/T)} = \frac{-\Delta H}{2.30R} - \frac{d \log_{10} K_m}{d(1/T)}$$

$$\frac{d \log_{10} \phi_s}{d(1/T)} = \frac{d \log_{10} K_m}{d(1/T)},$$

where ΔH represents the heat of reaction of Eq. (6) and R the gas constant. Then it follows that

$$\frac{d \log_{10} \phi_h + d \log_{10} \phi_s}{d(1/T)} = \frac{d \log_{10} K_h}{d(1/T)} = \frac{-\Delta H}{2.30R}.$$

Applying these equations to the data given in Fig. 3, we obtain the following figure:

$\Delta H = 14.9$ Kcal.

Some interesting inferrence may be made bassed on the finding that the reaction we have assumed: $E + H^+ \underset{K_h}{\leftrightarrows} EH^+$ shows the thermodynamical values pH 7.7 at 33° and $\Delta H = 14.9$ Kcal. The assumption that the form EH^+ is catalytically inactive implies that a certain chemical group in the enzyme molecule which can reversibly unite with hydrogen ion is essential for the enzyme activity. Comparison of the figures obtained above with those known for various groups in proteins allows us to infer that the essential group in question must be some amino group (see Table I.)

TABLE I

A Brief List of pK Values and Heats of Ionization (ΔH) of Acidic and Basic Groups Found in Proteins

Group	pK (25°)	ΔH
		Kcal/mole
Carboxyl	3.0— 4.7	±1.5
Phenolic-hydroxyly (tyrosine)	9.8—10.4	0.6
Sulfhydryl	9.1—10.8	—
Imidazolium (histidine)	5.6— 7.0	6.9— 7.5
Ammonium (α)	7.6— 8.4	10.0—13.0
Ammonium (α, cystine)	6.5— 8.5	—
Ammonium (ε, lysine)	9.4—10.6	10.0—12.0
Guanidinium (arginine)	11.6—12.6	12.0—13.0
The group in question of cholinesterase molecule.	7.7(33°) 8.0(25°)	14.9

This table was taken from Cohn and Edsall (*3*). The pK values given are those characteristic for the groups named, in peptides of known structure. The ΔH values are for both amino acids and peptides.

The fact that ϕ_h is increased with the increase of substrate concentration has lead us to the conclusion that the hydrogen ion and the substrate molecule compete with each other for the active group of the enzyme. We may, therefore, consider the property of the amino group in question as being such that it becomes repulsive for hydrogen ion when the enzyme combines with the substrate, while it hinders the combination of substrate molecule when it is in the ammonium form.

SUMMARY

1. Using the cholinesterase contained in equine serum, kinetic investigations were made on the relationships between the enzyme activity, hydrogen ion concentration, the concentration of substrate, and the temperature.

2. It was found that the hydrogen ion inhibits the enzyme action in competition with the substrate molecule.

3. It was shown that all the experimental data obtained could satisfactorily be explained by assuming the following reaction scheme:

$$E + S \rightleftarrows ES \quad \text{.....(I)}$$
$$ES \rightarrow E + P \quad \text{.....(II)}$$
$$E + H^+ \rightleftarrows EH^+ \quad \text{.....(III)}$$

where E is the free enzyme, S the substrate, ES the enzyme-substrate-complex, P the reaction products, and EH^+ the enzyme molecule rendered inactive by combination with hydrogen ion.

4. By analyses of the results obtained, the equilibrium constant and the heat of the reaction (III) were estimated to be $10^{-7.7}$ (mole/lit.) at 33° and 14.9 Kcal., respectively. Based on these figures it was inferred that a certain amino group in the enzyme molecule plays an important role in the catalytic action.

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STUDIES ON THE MECHANISN OF ACTION OF CHOLINESTERASE
II. EFFECTS OF FORMALDEHYDE, NITRITE AND OTHER CHEMICAL REAGENTS UPON THE ACTIVITY OF CHOLINESTERASE

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(Received for publication, January 16, 1952)

In the preceding paper of the author (*1*) a kinetic study was made on the effect of hydrogen ion upon the activity of cholinesterase in equine serum, and based on the data obtained it was inferred that a certain primary amino group in the enzyme molecule may play an essential part in its catalytic activity.

The experiments in this paper were performed with the view to get further information as to the nature of the active group or groups in the molecule of cholinesterase by investigating the effect of various substances which are known to affect specifically definite groupings in protein molecules. Further evidence for the essentiality of primary amino group will be adduced by showing that the enzyme activity is markedly affected by the action of formaldehyde and nitrite. The data will also be presented which indicate that, besides the amino group, a certain sulfhydryl group may bear an important part in the action of the enzyme.

METHOD

Using equine serum as the enzyme and acetylcholine chloride as the substrate, the activity was measured electrotitrimetrically as described in the previous paper. The methods of treatment of the enzyme with each chemical reagent will be described under the individual experiments.

RESULTS AND DISCUSSIONS

Effect of Formaldehyde—Formaldshyde is known to react readily with amino groups of proteins at its low concentration (*2*).

A half ml. of equine serum was mixed with 20 ml. of the formaldehyde solution of defidite concentration. The mixed solution was adjusted to a definite pH and left in a thermostat at 25° for 30 minutes. Then the substrate* was added and the reaction velocity was measured. The control experiment without the addition of formaldehyde was carried out under the same conditions. The results obtained are shown in Table 1, from which it can be seen that the cholinesterase activity was considerably reduced by formaldehyde under the experimental conditions used.

TABLE I

Effect of Formaldehyde upon the Cholinesterase (25°)

pH	Concentration of formaldehyde.	Activity of the treated serum represented in per cent of the control.
	per cent	*per cent*
7.0	1	66
	2	40
8.0	1	31
	2	24

Effect of Nitrite—It is known that nitrous acid deaminates aliphatic amino groups, reacts with phenol groups and also oxidizes such groups as sulfhydryl (*2*). According to Philpot *et al.* (*3*) and others (*2, 4, 5, 6*) the distinction between the deamination process and the reaction with tyrosine group can be made by the fact that in the presence of excess nitrite the former proceeds much more rapidly than the latter, and that the former is a second order reaction (in respect to the enzyme concentration), where as the latter is of the first order.

The equine serum was mixed with equal volume of acetate buffer (1 mole/lit.), the final pH of the mixture being 4.0. The mixture was cooled at 0°. After the addition of an equal volume of nitrite solution (2 mole/lit.) which had been cooled at 0°, 5 ml. each of the sample were removed at successive intervals and poured into 18 ml. of ice-cooled borate buffer (final pH 8) to stop the action of nitrous acid. Parallel with the above procedure, 5 ml. each of the sample were taken out and added to 18 ml. of the borate buffer saturated with hydrogen sulfide at 0°. The control without added nitrite was also run simultaneously and followed by the same procedure as the mentioned above

* The final concentration of acetylcholine chloride was $10^{-1.6}$ (mole/lit.)

to determine whether or not the enzyme was inactivated on standing at the acidity employed, and further to examine the effect of hydrogen sulfide upon the untreated enzyme. After leaving at 0° for about 3 hours, all these test and control semples ware dialyzed a gainst tap water for about 10 hours to remove the buffer and salts contained. The measurements of activities of these dialyzed samples were carried out at 20°, pH 7.5, using the substrate in concentration of $10^{-1.6}$ mole/lit.

A typical example of the experimental results obtained is shown in Fig. 1, in which the solid circles represent the activities of the enzyme treated with nitrous acid and then with hydrogen sulfide, and the open circles stand for those of the enzyme treated in the same way but without hydrogen sulfide, the reaction time being the time during which the enzyme had been in contact with nitrous acid.

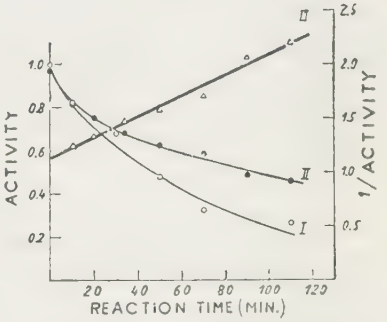

FIG. 1. Inactivation of cholinesterase by nitrous acid and subsequent reactivation with hydrogen sulfide.

I: Inactivation by nitrous acid. II: Inactivation by nitrous acid followed by treatment with hydrogen sulfide. III: The reciprocal of the activity in Curve II.

The control showed no inactivation on standing at pH 4.0 for the period examined and also no changes in the enzyme activity in the presence and absence of hydrogen sulfide, indicating that the inactivation observed with the test samples was solely due to the nitrous acid and that the reactivation with hydrogen sulfide was brought abuut by its restoring action upon the attacked groups of the nitrous acid-treated enzyme. This reactivation with hydrogen sulfide suggests the essentiality of the sulfhydryl group for the activity of this enzyme.

When the reciprocals of the activities observed with the samples which were reactivated with hydrogen sulfide, were plotted against the reaction time, the points fell on a straight line, as shown in Fig. 1, indicating that the reaction between the enzyme and the nitrous acid was of the second order (in respect to the concentration of enzyme) under the experimental conditions used. These observations may be taken as an additional evidence for the essentiality of the primary amino group in the activity of this enzyme.

Effect of Monoiodoacetate—The effect of iodoacetate is assumed to be specific for sulfhydryl groups of proteins (*2*). When the equine serum was treated with 0.1 mole/lit. iodoacetic acid at pH 7.0 for 30 minutes (25°), 84 per cent loss of activity was observed. This rapid inactivation of the enzyme appears to afford further evidence of the sulfhydryl group playing an important role in the activity of the enzyme.

Effect of Other Reagents—Effects of *p*-chloromercuri-benzoic acid* and iodine (*2*) upon the enzyme activity were examined with the purpose of obtaining additional evidence for the essentiality of the sulfhydryl group. It was, however, found that these chemical reagents did not cause any considerable loss of the enzyme activity under the recommended conditions (*2*, *6*). Further experiments with the purer enzyme preparation seem to be necessary to obtain any definite conclusion on the effects of these reagents.

SUMMARY

It was found that the activity of cholinesterase in equine serum was considerably suppressed by the effect of formaldehyde, nitrous acid, iodoacetic acid, but not by *p*-choromercuri-benzoic acid and iodine. Inhibitory effect of formaldehyde was attributed to its action upon some essential amino group in the enzyme molecule, the existence of which has already been inferred from the kinetic data on the effect of hydrogen ion upon the enzyme activity.

The inhibitory effect of nitrous acid was found to be partially removed by hydrogen sulfide. The residual inhibition was attributed to the reaction of nitrous acid with the primary amino group in question. The portion of the inhibition that was relieved by the action of hydrogen sulfide may be ascribed to the reaction of nitrous acid with a sulfhydryl group which may represent another essential structure in the enzyme molecule. The same group may also be the site of attack by monoidoacetic acid. Somewhat unintelligible was the finding that no inhibition was brought about by *p*-chloromercuri-benzoic acid and iodine, both of which are known to attack specifically sulfhydryl groups in protein molecule.

The author wishes to express his sincere gratitude to Prof. Hiroshi Tamiya and Dr. Atsushi Takamiya for their guidance in this work. These experiments

* This reagent is stated to attack most specifically sulfhydryl groups of proteins (*2*).

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STUDIES ON THE MECHANISM OF ACTION OF CHOLINESTERASE
III. KINETIC STUDIES ON THE INHIBITORY ACTION OF COUMARINE AND SEVERAL ALKYLAMMONIUM COMPONDS UPON CHOLINESTERASE

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(Received for publication, January 16, 1952)

In our preceding papers (*1*, *2*) the effects of several chemical substances and hydrogen ion upon the action of cholinesterase in equine serum was investigated, and based on the results obtained, some inferences were made on the nature of the essential chemical groups in the enzyme molecule. In order to get further insight into the nature of the active groups of the enzyme, kinetic studies were made on the inhibitory action of a lactone and several alkylammonium compounds which bear respectively a certain similarity in chemical structure to the substrate of the enzyme.

It was found that these substances compete with the susbtrate for the enzyme, and that, when they were added simdltaneously to the enzyme, there occured considerable interaction between them in their action upon the enzyme. Quantitative analysis of the data obtained has lead us to the conclusion that each of these substances attaches to a separate site on the molecule of the enzyme. It was suggested that the sites of combination of these substances may respresent the very points to which the substrate molecule will attach by virtue of its ester and ammonium groups, respectively, to form the intermediate substrate-enzyme complex. In our previous paper it was shown that the hydrogen ion also inhibits the enzyme activity in competition with the substrate. The site of attack by hydrogen ion was deduced to be a certain amino group, which may be different from, but structurally situating in close proximity to, the said two sites on the enzyme molecule. Indeed, it was found that there occurred also an interaction between hydrogen ion and lactone or alkylammonium compound in their action upon the enzyme.

METHOD

As in the previous works, equine serum was used as enzyme and acetylcholine as substrate. Inhibitors tested were choline chloride, trimethylethylammonium iodide, tetraethylammonium bromide,* beta-dimethylaminoethyl-benzhydryl ether hydrochoride,* and coumarin, the last one representing a lactone, while the others being alkylammonium compounds. The method adopted for the estimation of the reaction rate was the same as that described in the previous paper. Prior to the measurement of enzyme activity, the serum was mixed with varying concentrations of inhibitors and left in a thermostat at 33° for about 30 minutes in order to establish the equilibrium between the enzyme and the inhibitor.

RESULTS AND DISCUSSIONS

I. *Inhibitory Action of Coumarin*:

Coumarin** was found to inhibit the action of cholinesterase without being hydrolyzed by the enzyme as well as by hydroxyl ions under the experimental conditions employed.

Relationship between the Degree of Inhibition and the Concentration of Coumarin—When the degree of inhibition (H_c) was defined by

$$H_c=\frac{v_0-v}{v_0} \qquad \text{Eq. (1),}$$

where v and v_0 denote the reaction rate in the presence and absence, respectively, of coumarin, the relationship between H_c and the concentration of coumarin was found to be represented by the eatiquon:

$$H^c=\frac{[G_c]^2}{[G_c]^2+\phi^2} \qquad \text{Eq. (2),}$$

where $[G_c]$ indicates the concentration of coumarin and ϕ_c a constant which corresponds to the coumarin concentration causing 50 per cent inhibition. The fit of this equation to the experimental results is illustrated graphically in Fig. 1. As may be seen from the figure, the value of ϕ_c is a function of the concentration of substrate and hydrogen ion. When the logarithms of ϕ_c (at pH 8.0) was plotted against the logarithms

* These substances were kindly supplied by Kowa Kagaku Co., Ltd.

** Coumarin was dissolved in one per cent alcohol solution. It has been ascertained in our previous study that alcohol exerts no inhibitory action on the cholinesterase at this concentration.

of the substrate concentration (in mole/lit.), a straight line was obtained, which showed a slope approximately equal to 1/2 (see Fig. 2).

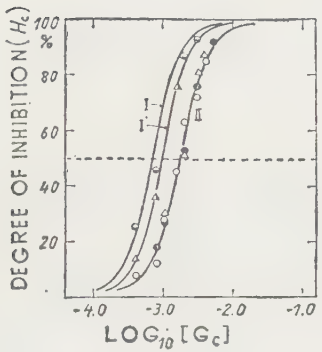

Fig. 1. Relationship between the degree of inhibition (H_c) and concentration of coumarin ([G_c]) under different experimental conditions (at 33°).

At the substrate concentration of $10^{-3.08}$ mole/lit.:

I (◒): pH 8.0; I' (▲): pH 6.0. At the substrate concentration of $10^{-2.38}$ mole/lit.:

II (○): pH 8.0; (●): pH 7.0; (△): pH 6.0 (----): Calculated according to Eq. (2).

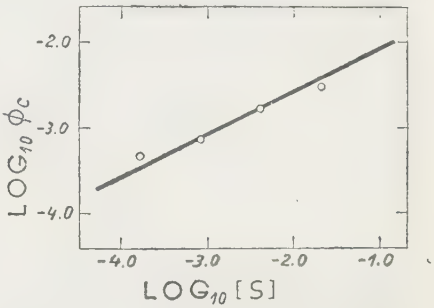

Fig. 2. Relationship between the value ϕ_c and substrate concentration [S] (at 33°, pH 8.0).

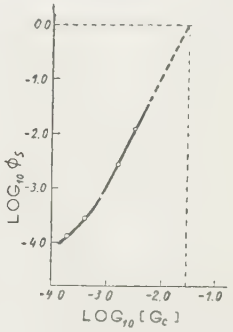

Fig. 3. Relationship between the Michaelis' constant ϕ_s and the concentration of coumarin [G_c] (at 33°, pH 8.0).

(○): Observed value. (----): Calculated value.

"*Activity-pS*" *Relationships in the Presence of Coumarin of Different Concentrations*—The activity-pS-relationship of the enzyme was modified by the effect of coumarin. Except at higher substrate concentrations and at higher concentrations of coumarin, the activity-pS relationship assumed the usual S-shaped curves, showing different values Michaelis' constant (ϕ_s) according to the concentration of the coumarin applied.

In Fig. 3, the logarithems of ϕ_s are plotted against the logarithms of the coumarin concentration. The linear portion of the curve showed a slope equal to 2.

The experimental results described above may be explained by assuming the following reactions:

$$\left.\begin{array}{l} E + S \underset{k_1}{\overset{k_1}{\leftrightarrows}} ES \\ ES \xrightarrow{k_3} E + P \end{array}\right\} \text{Eq. (3)},$$

$$E + 2G_c \underset{K_c}{\rightleftarrows} {}_EG_cG_c \qquad \text{Eq. (4)},$$

where E, S, P and G_c represent enzyme, substrate, reaction product and coumarin, respectively. Justification for assuming reaction of Eq. (*3*) has alreody been discussed in the previous study (1). By Eq. (4) we postulate that two molecules of coumarin will combine with a aolinesterase molecule in competition with the substrate. The value K_c indicated is the dissociation constant of the compound EG_cG_c assumed.

Bassed on these assumptions, the following relations may be derived for the stationary state of the reaction:

$$v = \frac{k_3\varepsilon}{1+\frac{K_m}{[S]}\left(1+\frac{[G]^2}{K_c}\right)} \qquad \text{Eq. (5)},$$

$$H^c = \frac{[G_c]}{[G]^2 + K_c\left(1+\frac{[S]}{K_m}\right)} \qquad \text{Eq. (6)},$$

where ε denotes the total concentration of the enzyme and K_m $(=k_2+k_3)/k_1)$ the Michaelis' constant. These equations indicate that the values ϕ_s and ϕ_c which had been determined experimentally should have the following implications:

$$\phi_s = K_m\left(1+\frac{[G_c]^2}{K_c}\right) \qquad \text{Eq. (7)},$$

$$\phi_c{}^2 = K_c\left(1+\frac{[S]}{K_m}\right) \qquad \text{Eq. (8)},$$

These equations are in good accord with the experimental results, except those at higher substrate concentration and higher concedtration of coumarin. Using Eq. (*7*) and Eq. (*8*) we can evaluate the value of K_c from the data given in Figs. 2 and 3, since K_m has already been determined in our previous work ($K_m = 10^{-4.2}$ (mole/lit.)). By extrapolating the linear portion of the curve in Fig. 3 toward $\log_{10}\phi_s = 0$, we

obtain $\log_{10} K_c = \log K_m + 2\log_{10}[G_c]$ at $\log_{10}\phi_{s=0} = -7.3$. Quite the same value of K_c was obtained by applying Eq. (8) to the data in Fig. 2. Close agreement between our deduction and the experimental data may bespeak the validity of our assumption that two molecules of coumarin attach to a cholinesterase molecule in competition with the substrate. Considering the structure of coumarin and acetylcholine it may be reasonable to assume that both these substances will attach, by virtue of their ester group, to the same structural group in the enzyme molecule.*

II. Inhibitory Action of Several Alkylammonium Compounds:

Relationship between the Degree of Inhibition H_a and Concentration of the Subetances—The relationship between the degree of inhibition and concentration of various alkylammonium compounds were scrutinized by the same procedure as described in the preceding section. The results obtained are summarized graphically in Fig. 4. In contrast to the case of coumarin, the following equation was found to fit in with the experimental results.

$$H_a = \frac{[G_a]}{[G_a] + \phi_a} \qquad \text{Eq. (9)},$$

where $[G_a]$ represents the consentration of the substance, and ϕ_a a ceartain constant which was different according to the sort of alkylammonium compounds.

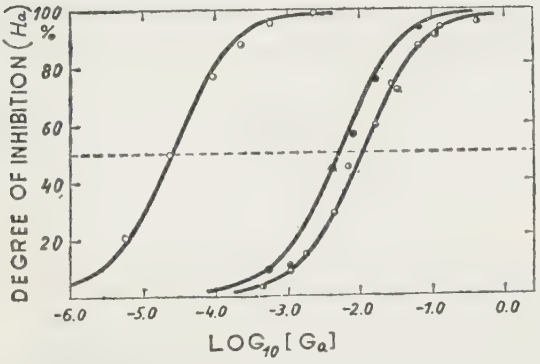

Fig. 4. Relationship between the degree of inhibition (H_a) and the concentration of ammonium compounds ($[G_a]$).

(○): β-Dimethylaminoethylbenzhydryl ether hydrochloride. (◑): Choline chloride. (●): Tetraethylammonium bromide. (◒): Trimethylethylammonium iodide. pH 8.0, 33°, substrate concentration $10^{-2.37}$ (mole/lit.).

* It has been concluded in our previous paper (*3*) that triacetin inhibits cholinesterase to some extent. It is conceivable that the ester also attaches by its -CO-O- group to the same site in the enzyme molecule.

Effect of Changes in the Substrate Concentration on the Value of ϕ_a—Also in this case the values of ϕ_a changed with the concentration of the substrate applied. As shown in Fig. 5, a plot of the logarithems of ϕ_a against the logarithems of substrate concentration gave a straight line with a tangent of 1.

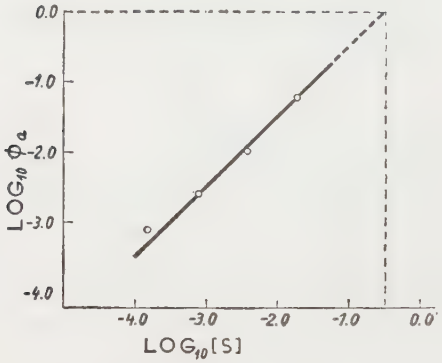

Fig. 5. Relationship between the ϕ_a-values of trimethylethylammonium iodide and substrate concentration (at 33°, pH 8.0).

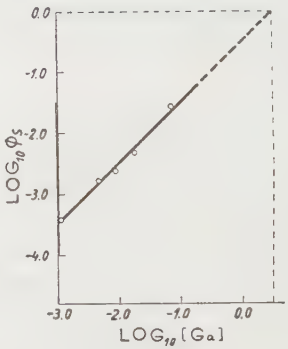

Fig. 6. Relationship between the Michaelis' constant ϕ_s and the concentration of trimethylethylammonium iodide (at 33°, pH 8.0).

"*Activity-pS*" *Relationships in the Presence of the Substance*—Addition of alkylammonium compounds also causes modifications in the "activity-pS" curve of cholinesterase (Fig. 6). By plotting the logarithems of ϕ_s values against the logarithms of the concentration of the substance, a straight line with a slope of unity was obtained.

To explain the data lescribed above, we assume the following reactions, which imply that the alkylammonium compound combines with the enzyme molecule by competing with the substrate molecule.

$$E + G_a \underset{K_a}{\rightleftarrows} EG_a \qquad \text{Eq. (10)}$$

$$E + S \underset{k_1}{\overset{k_2}{\rightleftarrows}} ES$$

$$ES \xrightarrow{k_3} E + P,$$

where G_a is the alkylammonium compound and K_a the dissociation constant of the compound EG_a. Stationary state kinetics based on the above scheme shows that the rate of enzymatic hydrolysis of acetylcholine v will be:

$$v = \frac{k_3 e}{1 + \frac{K_m}{[S]}\left(1 + \frac{[G_a]}{K_a}\right)} \qquad \text{Eq. (11),}$$

Accordingly,

$$H_a = \frac{[G_a]}{[G_a] + K_a\left(1 + \frac{[S]}{K_m}\right)} \qquad \text{Eq. (12),}$$

The implications of the values ϕ_s and ϕ_a are, therefore, as follows:

$$\phi_s = K_m\left(1 + \frac{[G_a]}{K_a}\right) \qquad \text{Eq. (13),}$$

$$_a = K\phi_a\left(1 + \frac{[S]}{K_m}\right) \qquad \text{Eq. (14),}$$

If $K_a \ll [G_a]$ and $K_m \ll [S]$, we many write

$$\phi_s = \frac{K_m}{K_a}[G_a] \qquad \text{Eq. (15),}$$

$$\phi_a = \frac{K_a}{K_m}[S] \qquad \text{Eq. (16),}$$

These relations are in good agreement with the experimental results. By applying Eq. 15 to the data given in Fig. 6, the value of K_m/K_a is estimated to be $10^{-0.48}$. Since K_m is known to be $10^{-4.2}$ (mole/lit.) we may evaluate K_a to be $10^{-3.7}$ (mole/lit.) in the case of trimethylammonium iodide. The same value of K_a could be obtained by applying Eq. (16) to the data given in Fig. 5. In Table I are summarized the values of K_a thus obtained for the various alkylammonium compounds examined.

TABLE I

Substance	K_a (33°)
	mole/lit.
Choline chloride	$10^{-4.1}$
Tetraethylammonium bromide	$10^{-3.7}$
Trimethylethylammonium iodide	$10^{-3.7}$
Beta-dimethylaminoethylbenzhydryl ether hydrochloride	$10^{-6.4}$

III. *Inhibitory Action of the Alkylammonium Compounds in the Presence of Other Substances*:

Interations among the Alkylammonium Compounds—It may be natural to assume that different alkylammonium compounds will attach to one and the same site on the enzyme molecule. If so, there will occur a competition between two alkylammonium compounds for that site when

they are simultaneously added to the enzyme solution. In this case we may assume the following reaction to take place:

$$E + G_{a1} \underset{K_{a1}}{\leftrightarrows} EG_{a1}$$
$$E + G_{a2} \underset{K_{a2}}{\leftrightarrows} EG_{a2}$$

where G_{a1} and G_{a2} denote different alkylamonium compounds.

Uuder such a circumstance, the stationary rate of the hydrolysis of acetylcholine will be represented by

$$v = \frac{k_3 \varepsilon}{1 + \frac{K_m}{[S]}\left(1 + \frac{[G_{a1}]}{K_{a1}} + \frac{[G_{a2}]}{K_{a2}}\right)} \qquad \text{Eq. (17),}$$

The degree of inhibition H_{a1} in respect to G_{a1} (in the presence of a definite concentration of G_{a2}) will be

$$H_{a1} = \frac{[G_{a1}]}{[G_{a1}] + K_{a1}\left(1 + \frac{[S]}{K_m} + \frac{[G_{a2}]}{K_{a2}}\right)} \qquad \text{Eq. (18),}$$

$$\phi_{a1} = K_{a1}\left(1 + \frac{[S]}{K_m} + \frac{[G_{a2}]}{K_{a2}}\right) \qquad \text{Eq. (19),}$$

Since K_m is known and K_{a1} and K_{a2} had already been determined separately for each alkylammonium compound, we can calculate the value ϕ_{a1} for any values of [S] and for any combinations of the compounds. The results of the experiments performed with choline-tetraethylammonium bromide and beta-dimethylaminoethylbenzhydryl ether-trimethylethylammonium iodide are shown in Fig. 7. In Table II are compared the experimental values of ϕ_{a1} with the values calcu-

TABLE II

	Substance	In the presence of	ϕ_a Observed	ϕ_a calculated
		mole/lit.	*mole/lit.*	*mole/lit.*
Curve I	Trimethylethyl-ammonium iodide	——	$10^{-1.98}$	——
Curve I′	,,	$10^{-4.3}$ β-dimethyl-aminoethylbenz-hydryl ether	$10^{-1.5}$	$10^{-1.4}$
Curve II	Tetraethyl-ammonium bromide	——	$10^{-2.5}$	——
Curve II′	,,	$10^{-2.8}$ Choline chloride	$10^{-2.1}$	$10^{-2.2}$

Temperature, 33°; pH 8.0; substrate concentration. $10^{-2.37}$ mole/lit. (Curve I and I′), $10^{-3.10}$ mole/lit. (Curve II and II′.).

lated by Eq. (19). The agreement between the two values may be regarded as satisfactory.

Interation between Coumarin and Alkylammonium Compound—In view of our inference that both coumarin and alkylammonium compound combine with enzyme in competition with the substrate, the question arises as to whether these two inhibitors attach to the same or different site on the enzyme molecule. Kinetical analysis of the phenomena observed when the two substances were added simultaneously showed that they attach to separate sites on the enzyme molecule. Interrelations between the substances and the enzyme molecule may be represented by the following scheme:

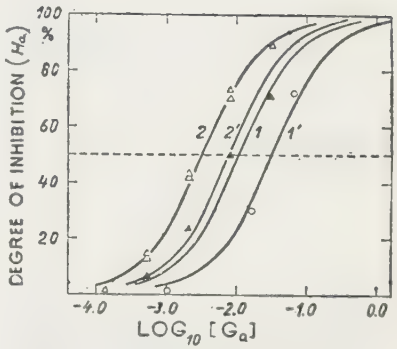

FIG. 7. H_a-log [G$_a$] relationship of alkylammonium compounds in the presence and absence of other alkylammonium compound. (For explanation see Table II).

$$E + G_a \underset{K_a}{\rightleftarrows} EG_a$$

$$E + 2G_c \underset{K_c}{\rightleftarrows} EG_cG_c$$

$$EG_a + 2G_c \underset{K_c'}{\rightleftarrows} EG_aG_cG_c \qquad \text{Eq. (20),}$$

$$(\text{or } EG_cG_c + G_a \underset{K_c'}{\rightleftarrows} EG_aG_cG_c) \qquad \text{Eq. (21),}$$

where $EG_aG_cG_c$ represents a compound composed of a molecule of the ammonium compound and two molecules of coumarin simultaneoulsy attached to an enzyme molecule. Based on these scheme, the following equations are derived.

$$v = \frac{k_3\varepsilon}{1 + \frac{K_m}{[S]} + \left(1 + \frac{[G_a]}{K_a} + \frac{[G_a]^2}{K_c} + \frac{[G_a]}{K_a}\cdot\frac{[G_a]^2}{K_c'}\right)} \qquad \text{Eq. (22),}$$

$$H_a = \frac{[G_a]}{[G_a] + K_a\left\{\frac{1 + \frac{[S]}{K_m} + \frac{[G_c]^2}{K_c}}{1 + \frac{[G_c]^2}{K_c'}}\right\}} \qquad \text{Eq. (23),}$$

$$\phi_a = K_a\left\{\frac{1+\frac{[S]}{K_m}+\frac{[G_c]^2}{K_c}}{1+\frac{[G_c]^2}{K_c'}}\right\} = K_a\left(1+\frac{[S]}{K_m}\right)\left\{\frac{\frac{K_c'/K_c-1}{1+\frac{[S]}{K_m}}}{\frac{K_c'}{[G_c]^2}+1}\right\} \qquad \text{Eq. (24),}$$

If the condition

$$\frac{K_c'/K_c}{1+\frac{[S]}{K_m}}-1=0 \qquad \text{Eq. (25),}$$

is satisfied, the value of ϕ_a will not be a function of the concentration of the coexisting coumarine, while it will increase or decrease with the increase of coumarin concentration according to the experimental conditions, $\frac{K_c'/K_c}{1+\frac{[S]}{K_m}} \gtrless 1$. The experimental results obtained were in good accordance with these predictions. Fig. 8 shows the experimental data obtained with trimethylethylammonium iodide. From the data of the experiment in which the condition, Eq. (25) was satisfied (see Curve 1), we may calculate the value of K'_c ($=10^{-5.47}$ mole/lit.) by substituting the known values of K_c, K_m and [S] into the equation. The ϕ_a value under other experimental conditions can be calculated by applying all these known values to Eq. (24). As may be seen from Table III the ϕ_a values thus evaluated for different substrate concentrations agree satisfactorily with the observed values.

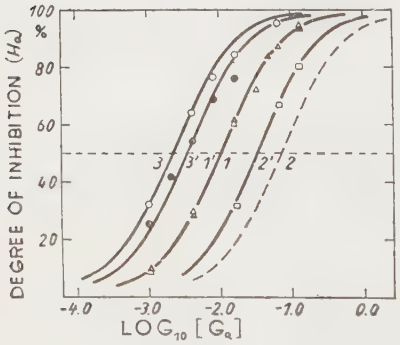

FIG. 8. H_a-log [G_a] relationship of trimethylethylammonium iodide in the presence and absence of coumarin (at 33°, pH 8.0). (For explanation see Table III).

Noteworthy is the fact that K_c' was found to be about seventy times larger than K_c. Exactly the same is true for K_a' in comparison with K_a when we assume the reaction of Eq. (21). That K_c' and K_a' are larger than K_c and K_a, respectively, was observed with all the alkylammonium compounds studied. Moreover the ratio of K_c'/K_c (K_a'/K_a) was found to be of the same order in all these cases. These facts may be interpreted

TABLE III

	Substrate concentration	Concentration of coumarin	ϕ_a Observed	ϕ_a Calculated
	mole/lit.	*mole/lit.*	*mole/lit.*	*mole/lit.*
Curve I (△)	$10^{-2.37}$	0	$10^{-1.98}$	——
Curve I′ (▲)	$10^{-2.37}$	$10^{-2.56}$	$10^{-1.98}$	$10^{-1.98}$
Curve II′ (□)	$10^{-1.63}$	$10^{-2.56}$	$10^{-1.48}$	$10^{-1.49}$
Curve II ( )	$10^{-1.63}$	0	$10^{-1.14}$*	——
Curve III (○)	$10^{-3.07}$	$10^{-2.56}$	$10^{-2.44}$	$10^{-2.11}$
Curve III′ (●)	$10^{-3.07}$	0	$10^{-2.64}$	——

* This was taken from the data shown in Fig. 5.

as indicating that coumarin and the alkylammonium compound exert a repulsive influence to each other, though they attach to different sites on the enzyme molecule.

IV. *Influences of Hydrogen Ions upon the Inhibitory Action of Coumarin and the Alkylammonium Compounds*:

In view of the fact found in the previous study (*1*), that the hydrogen ion, in a certain range of its concentration, inhibits the enzyme in competition with the substrate, it may be of interest to investigate the influence of pH on the inhibitory action of coumarin as well as of alkylammonium compounds.

Effect of pH upon the Action of Coumarin—The inhibition-log [G_c]-curves of coumarin obtained at pH 6 and 8 are already presented in Fig. 1. As is apparent from the figure, the form of the curve was not modified by the change in pH, while the value ϕ_c was affected by pH when the substrate concentration was $10^{-3.08}$ mole/lit. but remained unaffected when the substrate concentration was $10^{-2.38}$ mole/lit. These results can be explained by assuming the following reactions:

$$E + 2G_c \underset{K_c}{\rightleftarrows} EG_cG_c \qquad \text{Eq. (26)}$$

$$E + H^+ \underset{K_h}{\rightleftarrows} EH^+ \qquad \text{Eq. (26)}$$

$$EH^+ + 2G_c \underset{K_c'}{\rightleftarrows} EH^+G_cG_c \qquad \text{Eq. (27)}$$

$$(\text{or } EG_cG_c + H^+ \underset{K_h}{\rightleftarrows} EH^+G_cG_c) \qquad \text{Eq. (28)},$$

where K_h, K_h' and K_c' are the dissociation constants of the complexes assumed in the scheme. Based on these assumptions the degree of inhibition in respect to coumarin (H_c) is deduced to be

$$H_c=\frac{[G_c]^2}{\left\{\frac{\frac{K_h}{[H^+]}\left(1+\frac{[S]}{K_m}\right)+1}{\frac{K_h}{[H^+]}+\frac{K_c}{K_c'}}\right\}K_c+[G_c]^2} \qquad \text{Eq. (29),}$$

from which it follows that

$$\phi^2{}_c=K_c\left\{\frac{\frac{K_h}{[H^+]}\left(1+\frac{[S]}{K_m}\right)+1}{\frac{K_h}{[H^+]}+\frac{K_c}{K_c'}}\right\}$$

$$=K_c\left(1+\frac{[S]}{K_m}\right)\left\{1+\frac{\frac{K_c'/K_c-1}{1+\frac{[S]}{K_m}}}{\frac{K_h}{[H^+]}\cdot\frac{K_c}{K_c'}+1}\right\} \qquad \text{Eq. (30),}$$

This equation shows that in general ϕ_c increases with increase of hydrogen ion concentration, except under the condition in which the following ion concentration, except under the condition in which the following relation holds.

$$\frac{K_c'/K_c-1}{1+\frac{[S]}{K_m}}=0 \qquad \text{Eq. (31),}$$

This deduction agrees with our observation that, at the substrate concentration of $10^{-2.38}$ mole/lit., the value of ϕ_c was not modified by the change of hydrogen ion concentration in the range from 8 to 6, while at lower value of substrate concentration ϕ_c was increased with the increase of the hydrogen ion concentration. Applying Eq. (31) to the data obtained at the substrate concentration of $10^{-2.38}$ mole/lit., the value of K_c' was estimated to be $10^{-7.3}$ (mole/lit.). Applying this value of K_c' as well as other known constants to Eq. (30), the value of ϕ_c at the substrate concentration $10^{-3.08}$ mole/lit. and pH 6.0 was calculated to be $10^{-2.87}$ (mole/lit. which is in good agreement with the observed value $10^{-3.0}$ (mole/lit.). It should be noticed that K_c' is about sixty times larger than K_c. This fact indicates that coumarin and the hydrogen ion interact repulsively with each other in their reaction with the enzyme.

Effect of pH upon the Inhibitory Action of the Alkyl-Ammonium Compounds —Effect of hydrogen ion concentration upon the action of alklylammonium compounds was investigated in the pH range of 8 to 6. The experimental results obtained showed considerable disagreement with the expectation, as are shown in Fig. 9. Both with trimethylethylam-

monium iodide and choline chloride, the inhibition-log [G_a]-curve at pH 6.0 deviated markedly from Eq. (12), which was fulfilled at pH 8.0. If the alkylammonium compounds interact with hydrogen ion in a simple manner as was assumed for the case of coumarin or a competition is assumed to occure between them for the enzyme, the effect of hydrogen ions would be manifest only in the change of ϕ_a-value and not in the modification of the shape of the inhibition - log [G_a] - curve. The inhibition - log [G_a]-curve calculated for pH 6.0 on the latter assumption are represented by the dotted lines in Fig. 9, which deviate considerably from the observed curve. The question how to explain this phenomenon is left open to further investigations.

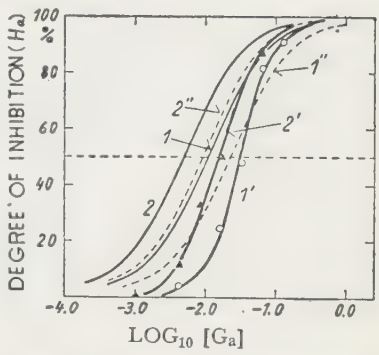

FIG. 9. H_a-log [G_a] relationships of trimethylethylammonium iodide and choline chloride at different pH (33°, substrate concentration $10^{-2.37}$ mole/lit.).

Trimethylethylammonium iodide: 1: pH 8.0. 1′: pH 6.0, observed. 1″: pH 6.0, calculated ($\phi_a = 10^{-1.63}$ mole/lit.).

Choline chloride: 2: pH 8.0. 2′: pH 6.0, observed. 2″: pH 6.0, calculated ($\phi_a = 10^{-2.03}$ mole/lit.).

SUMMARY

1. Inhibitory actions of coumarin and several alkylammonium compounds upon cholinesterase in equine serum were investigated by kinetical methods.

2. Effect of these substances is more or less decreased by the increase of substrate concentration, indicating that the substances compete with the substrate for the enzyme.

3. When two different alkylammonium compound were applied to the enzyme, they compete with each other for the enzyme. When coumarin and an alkylammonium compound were added to the enzyme, they can attach to the enzyme simultaneously, but there occurs a strong repulsive action between the substances in their combination with the enzyme.

4. It was discussed that coumarin and alkylammonium compound attach to separate sites in the molecule of the enzyme. In the reaction between the enzyme and the substrate (acetylcholine), the site having

affinity to coumarin may combine with the -CO-O- group and the site having affinity to alkylammonium compounds may combine with the alkylammonium group $N(R_3)^+$ of the substrate.

5. The efiects of coumarin and alkylammonium compounds upon the enzyme are interferred with by the action of hydrogen ion, which by itself inhibits the enzyme activity in competition with the substrate.

The authors wish to express thei gratitude to Prof. Hiroshi Tamiya for his encouragement and advice in this work. The work was supported by the Scientific Research Encouragement Grant and the Grant-in-aid for Private Research Institute donated by the Ministry of Education.

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KINETIC STUDIES ON THE ACTION OF GLUCOSE DEHYDROGENASE. II. SOME THERMODYNAMICAL AND KINETICAL QUANTITIES OF THE INTERMEDIATE REACTIONS

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It was previously reported (*2*) that the reaction catalysed by glucose dehydrogenase from *Aspergillus oryzae* may be represented—in so far as it occurs in the presence of sufficient amount of hydrogen acceptor—by the following sequence of reactions:

$$E+F \underset{k_1'}{\overset{k_1}{\rightleftarrows}} EF \qquad \text{Eq. (1),}$$

$$EF+S \underset{k_2'}{\overset{k_2}{\rightleftarrows}} EFS \qquad \text{Eq. (2),}$$

$$EFS \xrightarrow{k_3} E+P+H_2F \qquad \text{Eq. (3).}$$

(E: free enzyme molecule; F: hydrogen acceptor; S: the substrate; EF: a complex in which redox-dye is bound reversibly to E; EFS: another complex in which substrate is bound reversibly to EP; P: the product; H_2F: the leuco-dyestuff and k_1, k_1', $\cdots$: the rate constants.)

It has been discussed that the dissociation constant ($\boldsymbol{K}$) of the reaction of Eq. (2) may be computed by the following equation using the data obtained with two different hydrogen acceptors:

$$\boldsymbol{K}=\frac{\boldsymbol{V}_m^{(\mathrm{I})}\cdot\boldsymbol{M}^{(\mathrm{II})}-\boldsymbol{V}_m^{(\mathrm{II})}\cdot\boldsymbol{M}^{(\mathrm{I})}}{\boldsymbol{V}_m^{(\mathrm{I})}-\boldsymbol{V}_m^{(\mathrm{II})}}. \qquad \text{Eq. (4),}$$

where $\boldsymbol{V}_m$ is the maximum velocity to be attained at sufficiently high concentration of substrate, $\boldsymbol{M}$ the Michaelis constant and the superscripts I and II refer to different hydrogen acceptors.

In the present study we have investigated the effect of temperature upon the rate of over-all reaction using different substrates (D-glucose, D-xylose, D-galactose and D-mannose), and different hydrogen acceptors

and from the data obtained the enthalpy and the energy of activation of the reaction of Eq. (2) as well as the energy of activation of the reaction of Eq. (3), were estimated for each substrate and hydrogen acceptor.

METHOD

The enzyme was prepared from *Aspergillus oryzae* according to the method reported in the previous paper (*1*). As was described already, the rate of reaction was measured either by the Thunberg technique, iodometry or manometric method according as the sort of hydrogen acceptors applied. All experiments were carried out at pH 7.2.

RESULTS

D-Glucose as Substrate—In a previous paper (*2*) it was shown that the v/V_m-pS-curve obtained at 30° represented a typical "sygmoid curve of the first order." As is shown in Fig. 1, the same was found true at other temperatures, 1° and 20°. As may be seen from Table I, the values of V_m/ε and M changed with temperature. Noteworthy is the fact that with thionine or *p*-quinone as the hydrogen acceptor the value M increased with temperature, while the reverse was the case when 2,6-dichlorophenol-indophenol was used as hydrogen acceptor. Using the values of V_m/ε and M obtained for thionine and 2,6-dichlorophenol-

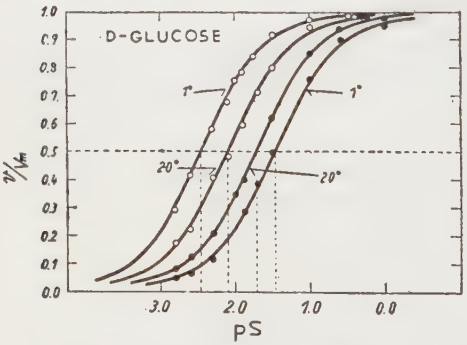

Fig. 1. The effect of temperature upon the v/V_m-pS-curve.

Substrate: d-glucose. —○—: Thionine was used as H-acceptor. —●—: 2,6-Dichlorophenol-indophenol was used as H-acceptor.

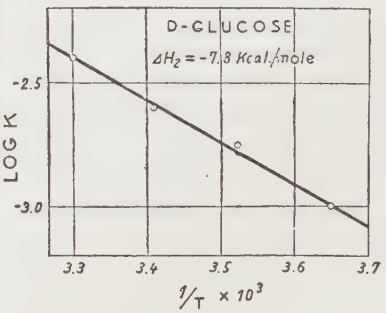

Fig. 2. The log K-1/T-relationship.

Substrate: D-glucose.

Table I

*The Values of V_m/ε, M and K for D-Glucose at Different Temperatures**

In the experiments with thionine and 2,6-dichlorophenol-indophenol; buffered enzyme solution (pH 7.2; 0.2 *mole/lit.* phosphate buffer), 0.5 ml.; redox-indicator ($2\times10^{-3.0}$ *mole/lit.*), 0.5 ml.; D-glucose solution, 1.0 ml. In the case of quinone: buffered enzyme solution (pH 7.2; 0.2 *mole/lit.* phosphate buffer), 1.0 ml.; quinone ($10^{-2.0}$ *mole/lit.*), 1.0 ml.; D-glucose solution, 3.0 ml.

	Temperature / Hydrogen acceptor	20°	10°	1°
$\frac{V_m/\varepsilon}{\left[\frac{mole}{lit.}\right]/\left[\frac{g.}{lit.}\right]\left[min.\right]}$	Thionine	$0.28\times10^{-4.0}$	$0.10\times10^{-4.0}$	$0.041\times10^{-4.0}$
	2,6-Dichlorophenol-indophenol	$1.06\times10^{-4.0}$	$0.723\times10^{-4.0}$	$0.48\times10^{-4.0}$
	p-Quinone	$2.9\times10^{-4.0}$	$1.3\times10^{-4.0}$	$0.62\times10^{-4.0}$
M $\left[\frac{min.}{lit.}\right]$	Thionine	0.0071	0.00465	0.0035
	2,6-Dichlorophenol-indophenol	0.020	0.0225	0.028
	p-Quinone			0.036
K $\left[\frac{min.}{lit.}\right]$		0.0025	0.0018	0.0012

* The values at 30° were summarized in Table II of J. *Biochem.* **39**, 37 (1952).

indophenol, the dissociation constant (K) at each temperature was calculated according to Eq. (4) and listed in Table I. When log K is plotted against 1/T, the points fall on a straight line over the temperature range of 1°-30° (see Fig. 2). From the tangent of this line the enthalph (ΔH_2) of the reaction of Eq. (2) is estimated to be—7.8 *kcal./mole.* On the other hand the free energy change (ΔF_2) of the same reaction is calculated from the value of K, and based on ΔH_2 and ΔF_2 the entropy change of the forward reaction was computed by

$$-\Delta H_2 = -\Delta F_2 - T\Delta S_2 . \qquad \text{Eq. (5)}$$

The values obtained are listed in Table VII.

As was discussed already, the value V_m/ε or the maximum reaction rate per unit concentration of enzyme is equal to k_3. Plot of log V_m/ε

versus 1/T gives straight lines, the tangents of which are different according to the hydrogen acceptors applied (see Fig. 3). From the tangents of these lines the energy of activation (E_3) of the reaction of Eq. (3) was calculated for each hydrogen acceptor. The values obtained are given in Table VIII.

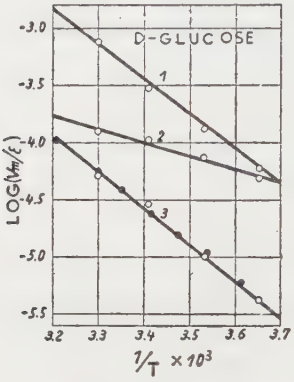

Fig. 3. Effect of hydrogen acceptors upon the log V_m/ε-1/T-relationship.

Substrate: D-Glucose. 1: *p*-Quinone was used as H-acceptor. 2: 2,6-Dichlorophenol-indophenol was used as H-acceptor. 3: Thionine was used as H-acceptor.* (* ○: The values obtained by the Thunberg technique. ●: The values obtained by manometric method.)

The kinetic considerations developed in the previous paper (*2*) showed that the Michaelis constant determined for our enzyme systems has the following implication

$$M=\frac{k'_2+k_3}{k_2} \qquad \text{Eq. (6),}$$

and therefore,

$$M-K=\frac{k_3}{k_2} \qquad \text{Eq. (7).}$$

Denoting

$$k_2=Z_2e^{-E_2/RT} \quad k_3=Z_3e^{-E3/RT} \qquad \text{Eq. (8),}$$

in which Z_2 and Z_3 are constants and E_2 and E_3 the activation energies of the reactions of Eq. (2) and Eq. (3), respectively, we have

$$-\frac{\mathrm{d}\ln(M-K)}{\mathrm{d}(1/T)}=\frac{E_3-E_2}{R} \qquad \text{Eq. (9).}$$

By plotting log $(M-K)$ against 1/T, we obtain straight lines showing different inclinations according to the hydrogen acceptors applied (see Fig. 4). From the tangents of these lines the value of E_3-E_2 was calculated to be 3.0 *kcal./mole* for thionine, −5.5 *kcal./mole* for 2,6-dichlorophenol-indophenol and 3.2 *kcal./mole* for *p*-quinone. Since E_3 is already known, we can obtain the value E_2 for each hydrogen acceptor. It should be remarked that the value E_2 is 11.0 *kcal./mole* in all cases, that

is, the activation energy of the reaction of Eq. (2) is independent of the hydrogen acceptors employed.

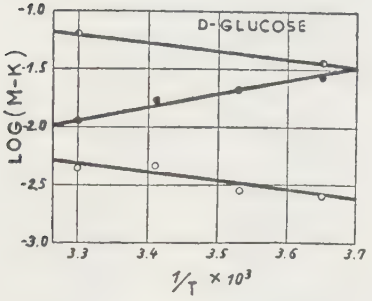

Fig. 4. The log $(M\text{-}K)$-1/T-relationship.

Substrate: D-glucose. —●—: 2,6-Dichlorophenol-indophenol was used as H-acceptor. —○—: Thionine was used as H-acceptor. —◐—: *p*-Quinone was used as H-acceptor.

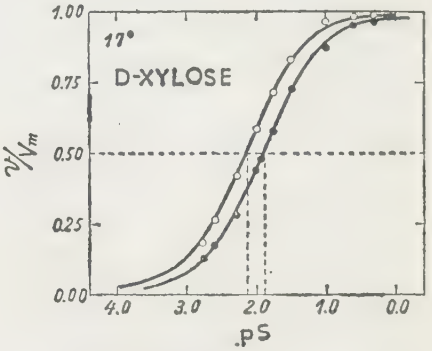

Fig. 5. The v/V_m-pS-curve of D-xylose.

—○—: Thionine was used as H-acceptor. —●—: 2,6-Dichlorophenol-indophenol was used as H-acceptor.

D-Xylose as Substrate—By using xylose as substrate, we obtained the v/V_m-pS-curve as shown in Fig. 5. Based on the values V_m/ε and M obtained at different temperatures (see Table II), thermodynamical quantities of the reaction of Eq. (2) as well as the activation energy of the reaction of Eq. (3) were calculated in the same manner as before. The values obtained are presented in Table VII and VIII.

The energy of activation of the reaction of Eq. (2) was calculated by using the data for thionine and 2,6-dichlorophenol-indophenol, and again it was found to be 11.0 *kcal./mole* in both cases.

D-Galactose and D-Mannose as Substrates—These two substrates appeared to have so little affinities to the enzyme, that the value V_m/ε could not be determined directly by experiment. Using the relationship:

$$v/\varepsilon = \frac{V_m/\varepsilon}{M/[S]+1} \qquad \text{Eq. (10),}$$

(v/ε: over-all velocity per unit concertration of enzyme observed when the substrate concentration is [S]),

Table II

The Values of V_m/ε, M and K for D-Xylose at Different Temperatures

In the experiments with thionine and 2,6-dichlorophenol-indophenol: buffered enzyme solution (pH 7.2; 0.2 *mole/lit.* phosphate buffer), 0.5 ml.; redox-indicator (2×10^{-3} *mole/lit.*), 0.5 ml.; D-xylose solution 1,0 ml. In the case of quinone: buffered enzyme solution (pH 7.2; 0.2 *mole/lit.* phosphate buffer), 1.0 ml.; quinone ($1.0^{-2.0}$ *mole/lit.*), 1.0 ml.; D-xylose solution 3.0 ml.

	Temperature / Hydrogen acceptor	30°	19°	17°	9°	1°
V_m/ε $\left[\frac{mole}{lit.}\right]\Big/\left[\frac{g.}{lit.}\right]\left[\frac{m.u.}{}\right]$	Thionine	$2.20\times10^{-5.0}$		$0.87\times10^{-5.0}$		$0.300\times10^{-5.0}$
	2,6-Dichlorophenol-indophenol	$3.00\times10^{-5.0}$		$1.9\times10^{-5.0}$		$0.914\times10^{-5.0}$
	p-Quinone	$2.5\times10^{-4.0}$	$1.1\times10^{-4.0}$		$0.4\times10^{-4.0}$	$0.20\times10^{-4.0}$
M $\left[\frac{mole}{lit.}\right]$	Thionine	0.0105		0.0077		0.00562
	2,6-Dichlorophenol-indophenol	0.0120		0.0130		0.0141
K $\left[\frac{mole}{lit.}\right]$		0.0064		0.0031		0.0015

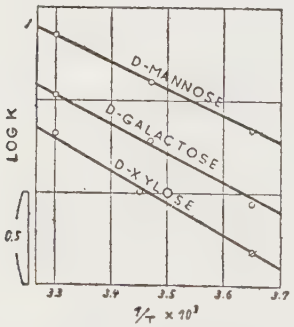

Fig. 6. The log K-1/T-relationships for various substrates.

the value of V_m/ε and M was determined by the method of least square. The values obtained are summarized in Table III and IV. Using these values, v/ε was calculated according to Eq. (10). In Table V and VI are shown to what extent the calculated values coincided with the observed ones.

Following the procedures decribed already, the values of ΔH_2, $\Delta F_2^{(303°K)}$, ΔS_2, E_2 and E_3 were calculated. The results obtained are listed in Tables VII and VIII.

TABLE III

The Values of V_m/ε, M and K for D-Galactose at Different Temperatures

Buffered enzyme solution (pH 7.2; 0.2 *mole/lit.* phosphate buffer), 0.5 ml.; redox-indicator ($2\times10^{-3.0}$ *mole/lit.*) 0.5 ml.; D-galactose solution, 1.0 ml.

	Hydrogen acceptor \ Temperature	30°	15°	1°
V_m/ε $\left[\frac{mole}{lit.}\right]/\left[\frac{g.}{lit.}\right]\left[min.\right]$	Thionine	$4.5\times10^{-5.0}$	$1.22\times10^{-5.0}$	$0.31\times10^{-5.0}$
	2, 6-Dichlorophenol-indophenol	$13.0\times10^{-5.0}$	$6.14\times10^{-5.0}$	$3.27\times10^{-5.0}$
M $\left[\frac{mole}{lit.}\right]$	Thionine	0.36	0.24	0.145
	2, 6-Dichlorophenol-indophenol	0.72	0.82	1.1
K $\left[\frac{mole}{lit.}\right]$		0.17	0.096	0.044

TABLE IV

The Values of V_m/ε, M and K for D-Mannose at Different Temperature

Buffered enzyme solution (pH 7.2, 0.2 *mole/lit.* phosphate buffer), 0.5 ml.; redox-indicator ($2\times10^{-3.0}$ *mole/lit.*), 0.5 ml.; D-mannose solution, 1.0 ml.

	Hydrogen acceptor \ Temperature	30°	15°	1°
V_m/ε $\left[\frac{mole}{lit.}\right]/\left[\frac{g.}{lit.}\right]\left[min.\right]$	Thionine	$2.16\times10^{-5.0}$	$0.64\times10^{-5.0}$	$0.155\times10^{-5.0}$
	2, 6-Dichlorophenol-indophenol	$4.48\times10^{-5.0}$	$2.56\times10^{-5.0}$	$1.1\times10^{-5.0}$
M $\left[\frac{mole}{lit.}\right]$	Thionine	0.71	0.50	0.32
	2, 6-Dichlorophenol-indophenol	1.09	1.4	1.60
K $\left[\frac{mole}{lit.}\right]$		0.36	0.20	0.107

Table V

The Rate at Various Concentration of D-Galactose

The calculated values are those obtained by Eq. (10) using the values of V_m/ε and M determined by the method of least square. Temperature; 30°

Concentration of D-galactose $\left[\frac{mole}{lit.}\right]$	Rate (v/ε) $\left[\frac{mole}{lit.}\right]/\left[\frac{g.}{lit.}\right]\left[min.\right]$			
	Thionine		2, 6-Dichlorophenol-indophenol	
	Observed values $\times 10^{-5.0}$	Calculated values $\times 10^{-5.0}$	Ovserved values $\times 10^{-5.0}$	Calculated values $10\times^{-5.0}$
1.00	3.1	3.3	7.23	7.56
0.50	2.7	2.6	5.75	5.3
0.25	1.85	1.85	3.6	3.5
0.125	1.35	1.2	1.95	1.9
0.100	1.0	1.0	1.5	1.6
0.05	0.55	0.55	0.86	0.85
0.033	0.38	0.38	0.57	0.57
0.025	0.27	0.28	0.43	0.43
0.0125	0.12	0.15	0.18	0.22

Table VI

The Rate at Various Concentrations of D-Mannose

The calculated values are those obtained by Eq. (10) using the values of V_m/ε and M determined by the method of least square. Temperature; 30°

Concentration of D-mannose $\left[\frac{mole}{lit.}\right]$	Rate (v/ε) $\left[\frac{mole}{lit.}\right]/\left[\frac{g.}{lit.}\right]\left[min.\right]$			
	Thionine		2, 6-Dichlorophenol-indophenol	
	Observed values $\times 10^{-5.0}$	Calculated values $\times 10^{-5.0}$	Observed values $\times 10^{-5.0}$	Calculated values $\times 10^{-5.0}$
1.00	1.26	1.26	2.16	2.16
0.50	0.90	0.90	1.48	1.4
0.25	0.56	0.56	0.83	0.83
0.125	0.32	0.32	0.47	0.47
0.10	0.28	0.27	0.36	0.39
0,05	0.14	0.14	0.18	0.195

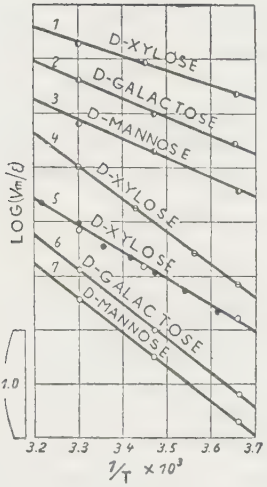

FIG. 7. Effect of hydrogen acceptors upon the log V_m/ε-1/T-relationship.

1, 2 and 3: 2,6-Dichlorophenol-indophenol was used as H-acceptor. 4: *p*-Quinone was used as H-acceptor. 5*, 6 and 7: Thionine was used as H-acceptor. (* ○: The values obtained by the Thunberg technique. ●: The values obtained by manometric method.)

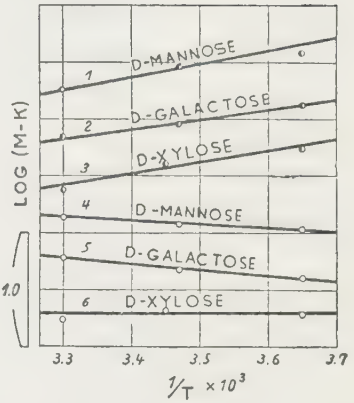

FIG. 8. The log $(M\text{-}K)$-1/T-relationships.

1, 2 and 3: 2,6-Dichlorophenol-indophenol was used as H-acceptor. 4, 5 and 6: Thionine was used as H-acceptor.

DISCUSSION

As may be seen from Table VIII, the value of E_2 for each substrate remained unchanged when different hydrogen acceptors were employed. This fact is of interest in connection with the observation made in our previous paper that the dissociation constant of the reaction of Eq. (2) was not affected by the difference in hydrogen acceptors applied. On the other hand, the activation energy in question was found to be dependent on the nature of substrate; namely 11.0 *kcal./mole* for glucose and xylose, 11.4 *kcal./mole* for galactose and 12.4 *kcal./mole* for mannose. Parallel with such a change, the enthalpy of the same reaction (ΔH_2) was fount to be -7.8 *kcal./mole* for glucose and xylose, -7.4 *kcal./mole* for galactose and -6.5 *kcal./mole* for mannose. This parallelism may be worthy of attention from the viewpoint of chemical kinetics.

TABLE VII

The Thermodynamical Quantities of the Reaction of Ep. (2), Each as a Function of the Substrate Applied

Substrate	ΔF_2 (303°K) $\left[\frac{K\ cal.}{mole}\right]$	ΔS_2 $\left[\frac{cal.}{deg \cdot mole}\right]$	ΔH_2 $\left[\frac{K\ cal.}{mole}\right]$
D-Glucose	−3.3	−15	−7.8
D-Xylose	−3.1	−16	−7.8
D-Galactose	−1.1	−20	−7.4
D-Mannose	−0.63	−20	−6.5

TABLE VIII

The Values of the Energy of Activation of the Reactions of Eq. (2) and Eq. (3) (at pH 7.2)

Substrate	Hydrogen acceptor	E_3 $\left[\frac{K\ cal.}{mole}\right]$	E_2-E_3 $\left[\frac{K\ cal.}{mole}\right]$	E_2 $\left[\frac{K\ cal.}{mole}\right]$
D-Glucose	Thionine	14.0	3.0	11.0
	2,6-Dichlorophenol-indophenol	5.5	−5.5	11.0
	p-Quinone	14.3	3.2	11.1
D-Xylose	Thionine	11.0	0	11.0
	2,6-Dichlorophenol-indophenol	6.5	−4.6	11.1
	p-Quinone	14.2		
D-Galactose	Thionine	14.3	2.8	11.5
	2,6-Dichlorophenol-indophenol	7.6	−3.7	11.3
D-Mannose	Thionine	14.7	2.3	12.4
	2,6-Dichlorophenol-indophenol	7.8	−4.6	12.4

The fact that the value V_m/ε varies both with substrates and with hydrogen acceptors shows that the rate constant (k_3) of the reaction of Eq. (3) is dependent on the properties of these two participant in the

reaction. Though the activation energy (E_3) of this reaction varied both with the substrates and with hydrogen acceptors, it seems to be more dependent on the sort of hydroygen acceptors than on the nature of substrate. Further systematic studies on these relations are desirable to get more incisive informations about the mechanism of the mutual reaction between substrate and hydrogen acceptor on the surface of the enzyme molecule.

SUMMARY

1. In continuation of the works previously reported, kinetic studies were made on the reaction catalysed by the glucose dehydrogenase isolated from *Aspergillus oryzae*.

2. For the reaction between the enzyme-acceptor-complex and the substrates some thermodynamical quantities (free engery, enthalpy and entropy) and the energy of activation were computed. The enthalpy and the energy of activation were found to be independent of the sort of the hydrogen acceptor applied, but decidedly dependent on the nature of substrate, varying in parallel relative to the sort of the substrates employed.

3. Estimation was also made for the energy of activation of the final step assumed in the over-all enzyme reaction, namely, the decomposition of the treble complex consisting of the enzyme, substrate and hydrogen acceptor. The value was found to be dependent considerably on the nature of hydrogen acceptor and also to some extent on the nature of the substrate applied.

In conclusion, the author wishes to express his thanks to Prof. H. Tamiya for his constant encouragement and suggestions during the course of this research.

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VITAMINS B_1 AND B_2 IN THE EGGS OF GIANT SALAMANDER AND SEA-TURTLE

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In order to investigate certain phenomena occurring in an organism of complicated structure, it is quite essential in many points to make a study, first of all, in case of a monocellular organism. From this viewpoint, an egg (embryo) is often chosen as the most favorable material for experiment. The present paper deals with the observations on the fluctuation in the contents of vitamins B_1 and B_2 in the eggs of giant salamander (*Megalobatrachus japonicus*, *Amphibia*) and sea-turtle (*Chelonia japonica*, *Reptilia*) at various stages of their development.

EXPERIMENTAL

Vitamin B_1 was estimated by A. Fujita's thiochrome fluorometric method (*1*), and vitamin B_2 by the lumiflavin method devised by the same author (*2*).

Giant Salamander Egg—Egg (*embryos*) used in the test were obtained twice in the upper stream of the Asahi River (Okayama Prefecture), and each time the estimation of vitamins were carried out promptly after the catch. The results obtained are shown in Table I.

Conceivably, vitamin B_1 and B_2 would not have been synthesized in egg during its developmental period, since there was hardly any difference in the total vitamin content between the two stages observed. But the percentage of free vitamin B_1 or B_2 was decreased, as embryos grew. This might be due to the situation that the vitamins are used for growth of embryo after being converted into their ester form. The very high concentration of vitamin B_2 was remarkable. On the other hand, no traces of the vitamins were found in the egg-enveloping liquid and sac.

TABLE I

Vitamins B_1 and B_2 in the egg of Giant Salamander

Developmental terms	Egg (Embryo)				Egg-enveloping liquid		Gelatinous sac	
	Average individual weight	Quantity of total $V.B_1$ / $V.B_2$	Concn. of total $V.B_1$ / $V.B_2$	Free $V.B_1$ / $V.B_2$ — Total $V.B_1$ / $V.B_2$	Average individual weight	$V.B_1$ / $V.B_2$	Average individual weight	$V.B_1$ / $V.B_2$
	g.	*γ*	*γ%*	*per cent*	*g.*		*g.*	
About 10 days (Sept. 10)	0.154	0.13	86.0	40	1.775	0	1.493	0
Beginning stage of development	Av. of 300 eggs	2.47	1602.0	95		0		0
About 34 days (Oct. 4)	0.229	0.17	75.2	28	4.662	0	0.725	0
Terminal stage of development	Av. of 120 embryos	2.61	1142.8	90		0		0

Sea-Turtle Egg—Eggs at five different stages of development were sent to the laboratory by rail from Hioki Seashore (Kagoshima Prefecture). A few of them, perhaps due to injury by hot weather during delayed transportation, showed some unreasonable values on estimation. But the results shown in Tables II and III may give due informations in certain respects.

Fluctuation in the Content of Vitamin B_1—The A-marked eggs were all laid by the same mother, and the B-marked were by another. The value of vitamin B_1 was nearly constant in B group especially at the early two stages, but there is difference between A and B groups in the content in egg-yolk. The total quantity of vitamin B_1 in an egg appeared to be variable according to the characteristics of its own mother, and its quantity at early stages was generally much less in egg-white than in egg-yolk. In either case, the quantity of free vitamin B_1 was predominant. The more development advanced, the more prominent became the concentration of total vitamin B_1 in egg-white. Since its amount was gradually decreased with the growth of egg, this increase seems to be due to simple concentration, but not to synthesis.

TABLE II

Vitamin B_1 in Sea-turtle Egg

Mother turtle	Days from spawned to captured (Development term)	Days from spawned to estimation	Vitamin B_1 in an egg except shell				Fraction of vitamin B_1				
			Av. wt. of an egg	Quantity of total V.B_1	Concn. of total V.B_1	Free V.B_1 / Total V.B_1	Fraction	Av. wt. of component	Quantity of total V.B_1	Concn. of total V.B_1	Free V.B_1 / Total V.B_1
			g.	*γ*	*γ%*	%		*g.*	*γ*	*γ%*	%
A	0 (July 2)	2 (July 4)	27.5	94.98	345	89	White	12.6	0.83	6.62	71
							Yolk	14.9	94.15	631.90	89
B	0 (July 12)	3 (July 15)	26.3	36.76	140	85	White	14.3	0.85	5.96	69
							Yolk	12.0	35.91	239.29	86
		5 (July 17)	25.9	37.13	143	89	White	13.9	1.46	10.56	82
							Yolk	12.0	35.67	297.28	89
	10 (July 22)	13 (July 25)	22.8	34.73	152	88	White	6.7	4.01	59.91	66
							Yolk	16.1	30.72	190.81	91
		15 (July 27)	22.1	48.56	220	68	White	6.8	1.77	26.10	53
							Yolk	15.3	46.79	305.84	68
	20 (Aug. 1)	26 (Aug. 7)	15.9	72.50	456	78	White	6.6	6.73	102.04	61
							Yolk and embryo	9.3	65.77	707.29	80
		29 (Aug. 10)	14.4	103.75	720	88	White	5.6	1.36	24.29	89
							Yolk and embryo	8.8	102.39	1163.47	88
	30 (Aug. 11)	32 (Aug. 13)	34.6	47.20	136	84	Except embryo	31.8	44.63	140.37	86
							Embryo	2.8	2.57	91.93	53
	40 (Aug. 21)	42 (Aug. 23)	35.0	46.82	134	84	Except embryo	27.3	34.63	126.86	65
							Embryo	7.7	12.19	158.40	60

Vitamin B_1 content of egg-yolk was almost constant until ten or fifteen days of development, but after twenty days it increased suddenly,

and gave an impression as if vitamin B_1 synthesis had occurred. However, there was some doubt that such a high value might be caused by a spoiling of experimental material as above-mentioned. At the terminal two stages, vitamin B_1 of an egg gradually became to be concentrated into embryonal part, where the content of ester form of vitamin B_1 was predominant.

Fluctuation in the Content of Vitamin B_2—Vitamin B_2 occurred in egg-yolk as well as in egg-white. In the former it existed more in combined form than in free state, whereas the reverse was the case with the latter.

TABLE III

Vitamin B_2 in Sea-turtle Egg

Days from spawted to captured (Developmental term)	Days from spawned to estimation	Vitamin B_2 in an egg except shell				Fraction of vitamin B_2				
		Av. wt. of an egg	Quantity of total V.B_2	Concn. of total V.B_2	Free V.B_2 / Total V.B_2	Fraction	Av. wt. of component	Quantity of total V.B_2	Concn. of total V.B_2	Free V.B_2 / Total V.B_2
		g.	*γ*	*γ%*	%		*g.*	*γ*	*γ%*	%
0 (July 12)	3 (July 15)	26.3	49.91	189	35	White	14.3	108.51	15.51	73
						Yolk	12.0	286.72	34.40	18
10 (July 22)	14 (July 26)	22.8	50.89	223	28	White	6.7	97.43	6.52	69
						Yolk	16.1	275.61	44.37	22
20		—	—	—	—	—	—	—	—	—
30 (Aug. 11)	32 (Aug. 13)	34.6	111.84	323	52	Except embryo	31.8	345.12	109.74	54
						Embryo	2.8	75.31	2.10	11
40 (Aug. 21)	42 (Aug. 23)	35.0	115.77	330	46	Except embryo	27.3	281.22	76.77	64
						Embryo	7.7	506.54	34.00	13

Total vitamin B_2 content in the eggs at the two end stages was found to be about twice as much as it was at the beginning, but as to synthesis of vitamin B_2 nothing can be said for the same reason as in the case of vitamin B_1. In embryo vitamin B_2 also seems to be concentrated during the growth, existing chiefly in the ester form.

SUMMARY

1. Studies have been made on the fluctuation of vitamins B_1 and B_2 contents in giant salamander and sea-turtle eggs during their development.

2. In giant salamander eggs no synthesis of vitamins B_1 and B_2 are likely to take place during its growth process. The very high concentration of vitamin B_2 is remarkable. No traces of the vitamins were found in the egg-enveloping liquid and sac.

3. In sea-turtle eggs the content of vitamin B_1 seems variable according to its own mother. The quantity of vitamin B_1 is generally found more in egg-yolk than in egg-white, and free vitamin is dominant. Vitamin B_2 occurs in both egg-yolk and white. In the former it is poor in free form, while, in the latter it is comparatively rich. In the intermediate period of development the synthesis of B_1 and B_2 might take place, but a further investigation is necessary to verify this point.

4. In both giant salamander and sea-turtle eggs, vitamins B_1 and B_2 became concentrated as embryo grew, and concomitantly the amounts of their ester form became more pronounced.

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BEHAVIOR OF MICROORGANISMS TOWARDS MESO-DIAMINOSUCCINIC ACID

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Biological significance of C_4-dicarboxylic acids, *i.e.*, succinic, fumaric, malic, aspartic, tartaric, *etc.*, are well known. Diaminosuccinic acids, however, have not yet been found in nature and so have never been fully investigated. The present authors, with biochemical interest on the amino acids, have studied the behavior of microorganisms towards meso-diaminosuccinic acid (m-DASA).

EXPERIMENTAL

Growth of Microorganisms in Media Containing m-DASA—Some microorganisms were able to grow in the following media containing m-DASA as a sole source of nitrogen. Table I shows the result of culture using *Echerichia coli communis*, *Pseudomonas aeruginosa*, *Bacterium erythrogenes*, *Aspergillus oryzae*, *A. niger and Streptomyces griseus.*

In this culture, ammonia and α-ketoacid, possible products of oxidative deamination, could not be detected. When L-leucine was used as N-source in place of m-DASA, considerable quantities of ammonia and α-ketoisocaproic acid were obtained.

Deamination of m-DASA by Resting Cells—The oxidative deamination, the most common process of amino acid breakdown, was tested first. When resting cells were incubated at 38° for 3 hrs. with L-leucine, oxidative deamination was observed. However, when the substrate was replaced by m-DASA, no oxidative process was observed (Table II).*

On the other hand, a slight production of fumaric acid and hydrazine was observed with the resting cells of *E. coli*, a fact indicating the oc-

* It was also confirmed that D-amino acid oxidase was inactive to m-DASA. In this experiment, FAD and toe apoenzyme from pig kidney were used.

Table I

Growth of Microorganisms in Media Containing m-DASA as a Sole Source of Nitrogen

Organisms	Medium	Growth after 4 days	pH	
			Initial	Final
E. coli	A	+ (anaerobic)	8.0	8.2
,,	A	− (aerobic)	—	—
P. aeruginosa	A	+ (,,)	8.0	9.0
B. erythrogenes	A	++ (,,)	8.0	8.5
A. oryzae	B	+ (,,)	6.0	6.4
A. niger	B	+ (,,)	6.0	5.0
S. griseus	B	+ (,,)	6.0	7.2

++ normal growth, + slight growth, − no growth.

Medium A: m-DASA 1.0 g, glycerol 50 ml, Na_2CO_3 1.7 g, NaCl 2.3 g, Na-citrate 5.0 g, KH_2PO_4 3.0 g, $CaCO_3$ 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.8 g, $FeCl_3$ trace, H_2O 1000 ml, pH 8.0, 38°.

Medium B: m-DASA 1.0 g, glucose 7.5 g, KCl 0.5 g, KH_2PO_4 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCO_3$ 0.5 g, $FeCl_3$ trace, H_2O 1000 ml, pH 6.0, 25°.

Table II

Tests of Oxidative Deamination of m-DASA by Resting Cells[1]

Substrate	m-DASA 0.1% (suspension)[2]			L-Leucine 0.1%		
Organisms	O_2 Uptake	NH_3 increase	Ketoacid detected	O_2 Uptake	NH_3 increase	Ketoacid detected[3]
	cu. mm.			*cu. mm.*		
E. coli	0	—	none	70	+	α-ketoiso-caproic acid
A. oryzae	0	—	none	60	+	,,
S. griseus	0	—	none	65	+	,,

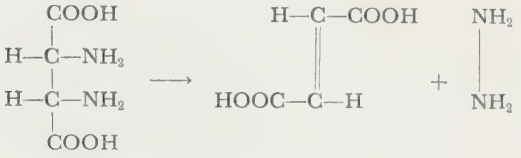
1) Cells were harvested and suspended in 1/15 *M* phosphate, pH 7.5.

2) Solubility of m-DASA is 1/300 *M* in water.

3) Ketoacid was identified by paper chromatography of 2,4-dinitrophenylhydrazone (*1*).

currence of an aspartase-like deamination:**

** Jacobsohn (1936) (*2*) assumed the reverse reaction, the addition of hydrazine to fumaric acid.



m-DASA was incubated in the presence of toluene with resting cells of *E. coli*, which were grown for 16—20 hours, at 30° on broth-agar, harvested, centrifuged and washed three times with distilled water. m-DASA was suspended (1/100 *M*) in 1/15 *M* phosphate buffer, pH 7.4. The reaction mixture was kept at 38° for 24 hours. Then it was deproteinized with trichloracetic acid and centrifuged. 10 per cent of sulfuric acid was added to the supernatant and the mixture extracted with ether. The ether extract was evaporated. The residue could reduce permanganate and bromine solution, and it was confirmed by paper chromatography (*3*) that the most part of the residue was fumaric acid. On the other hand, the supernatant was made alkaline with KOH and warmed with mercuric chloride. Mercury was reduced. *p*-dimethylaminobenzaldehyde reagent (*4*) gave an orange coloration of azine. These facts show the existence of hydrazine. The concentration of hydrazine was always lower than that of fumaric acid. It might be due to the further decomposition.

Inhibition of Bacterial Growth by m-DASA—The authors, studying with *E. coli* and *A. vinelandii*, found that the growth of them was inhibited by m-DASA. If *E. coli* was cultured in a medium with aspartic acid as a sole source of carbon and nitrogen (KH_2PO_4 0.04 g, Na_2CO_3 0.02 g, $MgSO_4 \cdot 7H_2O$ 0.01 g, $FeCl_3$ trace, NaCl 0.02 g, H_2O 1000 ml, Aspartic acid 0.01 *M*, pH 7.4, 25°) an inhibition of growth was observed by the addition of 1/300 *M* m-DASA (Fig. 1).

When aspartic acid was replaced by glutamic acid (0.01 *M*), no inhibition was observed (Fig. 3). The growth however was also inhibited by 1/300 *M* m-DASA in the medium with NH_4 Cl (0.01 *M*) and C-source (0.01 *M*) (*i.e.*, oxaloacetic acid, α-ketoglutaric acid, malic acid, fumaric acid, succinic acid, citric acid, glycerol, glucose) instead of aspartic acid or glutamic acid. Quite similar inhibitory effects were observed also by the addition of 1.5×10^{-7} *M* hydrazine sulfate Figs. 2 and 3).

Growth of *Azotobacter vinelandii* (5), which has a nitrogen fixing power, was also inhibited by 1/100 *M* m-DASA and by 1.5×10^{-7} *M*

hydrazine sulfate in the Ashby's medium (Mannitol 2g, NaCl 0.2g, KH_2PO_4 0.2g, $CaSO_4$ 0.1g, $MgSO_4 \cdot 7H_2O$ 0.2g, $FeCl_3$, Na_2MoO_4, trace, H_2O 1000ml, pH 7.2, 25°.) in the same manner (Figs. 4 and 5).

This inhibition could, however, be removed by the addition of 1/50 *M* ammonia in both cases (Fig. 6). It may be considered that the inhibitory action of m-DASA is due to the hydrazine formed by its enzymatic decomposition.

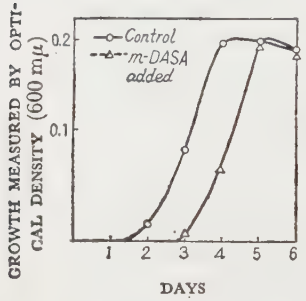


Fig. 1. Growth of *E. coli* in the medium of aspartic acid with or without m-DASA.

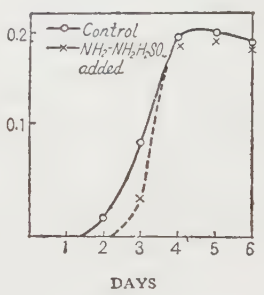


Fig. 2. Growth of *E. coli* in the medium of aspartic acid with or without hydrazine.

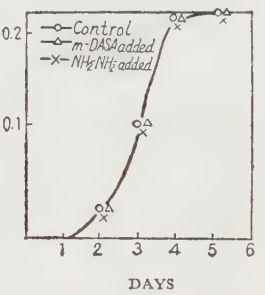


Fig. 3. Growth of *E. coli* in the medium of glutamic acid.

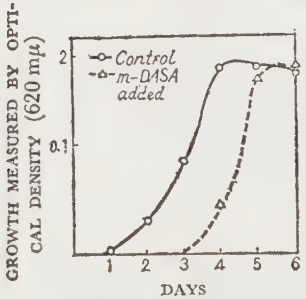


Fig. 4. Growth of *A. vinelandii* in the Ashby's medium with or without m-DASA.

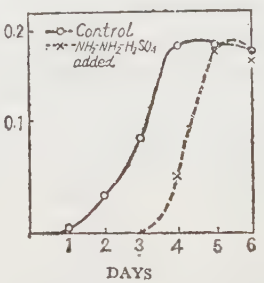


Fig. 5. Growth of *A. vinelandii*, in the Ashby's medium with or without hydrazine.

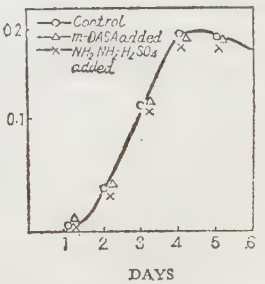


Fig. 6. Growth of *A. vinelandii* in the Ashby's medium with 1/50 *M* NH_4^+.

SUMMARY

1. The authors studied the behavior of microorganisms towards meso-diaminosuccinic acid (m-DASA). The amino was not an adequate

nutrient for microorganisms so far tested except for a strain of *B. erythrogenes*.

2. No oxidative breakdown of m-DASA could be observed.

3. The deamination forming fumaric acid and hydrazine was observed with resting cells of *E. coli*.

4. Effects of m-DASA and hydrazine on the growth of *E. coli* and *A. vinelandii* were studied under various conditions. Inhibitory action of m-DASA observed in certain cases may be attributed to the action of hydrazine formed from m-DASA.

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KINETIC STUDIES ON THE ACTION OF GLUCOSE DEHYDROGENASE III. COMPETITIVE INTERACTION BETWEEN TWO SUBSTRATES IN THEIR REACTION WITH THE ENZYME MOLECULE*

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(Received for publication, February 9, 1952)

Previous works (*1, 2, 3, 4, 5*) have shown that our sample of glucose dehydrogenase isolated from *Aspergillus oryzae* oxidized D-glucose, D-galactose, D-mannose, D-xylose and 6-phospho-D-glucose, but not D-arabinose, L-rhamnose and α-methyl-glucoside. In the present paper are described the experiments which were performed to make clear the mutual effects between two oxidizable substrates or between oxidizable and non-oxidizable substances in their reaction with the enzyme molecule. It was revealed that the oxidizable substrates, with the exception of 6-phospho-D-glucose, act competitively with each other, and are, therefore, conceivably dealt with by the same active group in the enzyme molecule. Among the non-oxidizable substances, only D-arabinose was shown to inhibit competitively the oxidation of the usable substrates. The oxidation of 6-phospho-D-glucose was attributed to the action of some specific enzyme which might have existed as an impurity in the enzyme preparation.

METHODS

The enzyme was isolated from *Asp. oryzae* and purified as described previously (*2, 3*). Using the Thunberg technique, the oxidation rate of the substrates was measured by the time required to bring about 90 per cent decolorization of the redox-dye (*cf.*, *4*). The reaction mixtures used were of the following composition: 0.5 ml. of buffered enzyme solution (0.1 *mole/lit.* phosphate buffer, pH 7.2) were placed in the main tube, and 0.5 ml. of a substrate solution, 0.5 ml. of another substrate solution (or poison), and 0.5 ml. of $2\times10^{-3.0}$ *mole/lit.* solution of redox-

* The first report on this work was made at the monthly meeting of the Botanical Society of Japan held in Tokyo in December, 1943.

indicator (thionine or 2,6-dichlorophenol-indophenol) in the side bulb. All the experiments were carried out at pH 7.2 and at 30°.

THEORETICAL CONSIDERATION

Before presenting experimental results, it seems advisable to give some theoretical considerations dealing with the phenomena in general to be expected in the simultaneous presence of two substrates.

Let us consider that the two substrates (S_a and S_b) are attacked by one and the same enzyme, and that they behave competitively with each other in their reaction with the enzyme molecule. On the basis of the kinetic scheme previously proposed (*4*), the following reactions will be assumed to occur in the simultaneous presence of the two substrates:

$$E+F \underset{k'_1}{\overset{k_1}{\rightleftarrows}} EF \qquad \text{Eq. (1)},$$

$$EF+S_a \underset{k'_{2,(a)}}{\overset{k_{2,(a)}}{\rightleftarrows}} EFS_a \qquad \text{Eq. (2)},$$

$$EF+S_b \underset{k'_{2,(b)}}{\overset{k_{2,(b)}}{\rightleftarrows}} EFS_b \qquad \text{Eq. (3)},$$

$$EFS_a \xrightarrow{k_{3,(a)}} E+H_2F+P_a \qquad \text{Eq. (4)},$$

$$EFS_b \xrightarrow{k_{3,(b)}} E+H_2F+P_b \qquad \text{Eq. (5)},$$

where E is the free molecule of the dehydrogenase, F the hydrogen acceptor, S_a and S_b the molecules of substrates, EF, EFS_a and EFS_b the complexes formed of the molecules indicated, and P_a and P_b the products. The value k assigned to each formula means the rate constant of each reaction.

If the overall rate of the reduction of H-acceptor is denoted by $v_{a,b}$ then,

$$v_{a,b}=-\frac{d[F]}{dt}=k_{3,(a)}[EFS_a]+k_{3,(b)}[EFS_b].$$

At stationary state we have

$$v=\frac{\frac{[S_a]}{M^{(a)}}V_m^{(a)}+\frac{[S_b]}{M^{(b)}}V_m^{(b)}}{1+\frac{S_a}{M^{(a)}}+\frac{S_b}{M^{(b)}}} \qquad \text{Eq. (6)},*$$

* This equation was independently obtained by Thorn (*6*) and modified by Whittaker and Adams (*7*).

where V_m is the maximum velocity of the overall reaction to be observed in the presence of a single substrate at saturating concentration, M the Michaelis constant, and the superscripts a and b refer to different substrates. In the presence of S_a alone, the velocity (v_a) will be, as previously described,

$$v_a = \frac{\frac{[S_a]}{M^{(a)}} V_m^{(a)}}{1 + \frac{[S_a]}{M^{(a)}}} \qquad \text{Eq. (7).}$$

If the value of $V_m^{(b)}/\varepsilon$ (ε being the total concentration of the enzyme) is considerably smaller than that of $V_m^{(a)}/\varepsilon$ and $M^{(b)}$ is not too large, it may be expected that the oxidation of S_a will be apparently inhibited by S_b, provided that the given concentration of the former (S_a) is higher than the value of $(V_m^{(b)} M_m^{(a)})/(V_m^{(a)} - V_m^{(b)})$.

When the concentration of S_b is varied, while that of S_a is kept constant, the degree of inhibition ($H_{\bar{a},b}$) referred to S_b may be defined by

$$H_{\bar{a},b} = 1 - \frac{v_{\bar{a},b} - V_m^{(b)}}{v_{\bar{a}} - V_m^{(b)}} \qquad \text{Eq. (8),}$$

where the subscript a means the presence of S_a at a constant concentration. Taking into consideration the reaction given above, we have

$$H_{\bar{a},b} = \frac{[S_b]}{\phi_{\bar{a},b} + [S_b]} \qquad \text{Eq. (9)}$$

where

$$\phi_{\bar{a},b} = M^{(b)}(1 + [S_a]/M^{(a)}). \qquad \text{Eq. (10).}$$

Eq. (9) shows that the $H_{\bar{a},b}$-pS_b-relationship will give a "sigmoid curve of the first order" (*cf.* 8).

When a poison (G) which causes the competitive inhibition is substituted for S_b, the following reaction will occur besides the reactions of Eqs. (1), (2), and (4),

$$EF + G \underset{K_G}{\rightleftharpoons} EFG \qquad \text{Eq. (11)}$$

where K_G represents the dissociation constant of this reaction. In this case, he degree of inhibition (H) caused by G is given by

$$H = 1 - \frac{v_{\bar{a}G}}{v_{\bar{a}}} = \frac{[G]}{\phi + [G]} \qquad \text{Eq. (12),}$$

where

$$\phi = K_G\left(1 + \frac{[S_a]}{M^{(a)}}\right) \qquad \text{Eq. (13),}$$

and $v_{\bar{a},G}$ is the velocity of the overall reaction in the presence of the competitive poison. The H-pG-relationship will be represented also by a sigmoid curve of the first order.

If the two substrates (or the poison and the substrate) do not act competitively with each other, the relations to be found will be entirely different from those given above. When the two substrates are dealt with by different specific enzymes, the rate of the reaction defined by $v_{a,b}$ will always be equal to the sum of the rate referring to each substrate alone, namely:

$$v_{a,b}=v_a+v_b. \qquad \text{Eq. (14).}$$

RESULTS

I. *Competition between D-Xylose and Other Oxidizable Sugars.*

(a) *D-Glucose vs. D-Xylose*—For these sugars the following values have been obtained (at pH 7.2 and 30°) in our previous experiments (*4*, *5*).

$$V_m^{(a)}/\varepsilon=5.0\times10^{-5\cdot0}\left[\frac{mole}{lit.}\right]\Big/\left[\frac{g.}{lit.}\right]\left[min.\right]$$
$$M^{(a)}=0.0085\quad\left[\frac{mole}{lit.}\right]$$

For D-glucose (H-acceptor: thionine.)

$$V_m^{(b)}/\varepsilon=2.2\times10^{-5\cdot0}\left[\frac{mole}{lit.}\right]\Big/\left[\frac{g.}{lit.}\right]\left[min.\right]$$
$$M^{(b)}=0.0105\quad\left[\frac{mole}{lit.}\right]$$

For D-glucose (H-acceptor: thionine.)

Using the same H-acceptor, the rate of the reaction ($v_{a,b}/\varepsilon$) in the simultaneous presence of the two sugars was measured and the values obtained were compared with those calculated by Eq. (6). As may be seen from Table I, the agreement between the observed and calculated values was quite satisfactory. According to Eq. (8) the degree of the inhibition of glucose-oxidation caused by xylose was calculated. On plotting $H_{\bar{a},b}$ against pS_b ($=-\log_{10}[S_b]$), we obtain a sigmoid curve as shown in Fig. 1. The observed values of $\phi_{\bar{a},b}$, *i.e.* the concentrations of xylose at $H_{\bar{a},b}=0.5$, were 0.073 *mole/lit.* at $[S_a]=0.050$ *mole/lit.* and 0.040 *mole/lit.* at $[S_a]=0.025$ *mole/lit.*, respectively. As is shown in Table III, these values coincided well with those calculated by Eq. (10).

When 2,6-dichlorophenol-indophenol was used as a hydrogen acceptor, an $H_{\bar{a},b}$-pS_b-relationship as is illustrated in Fig. 2 was obtained.

TABLE I

The Rate of Reaction ($v_{a,b}/\varepsilon$) in the Simultaneous Presence of D-Glucose (S_a) and D-Xylose (S_b)

H-Acceptor: thionine. Temperature: 30°. pH: 7.2.

Substrate	Concentrations of substrates (*mole/lit.*)	Rate $(v_{a,b}/\varepsilon)\left[\frac{mole}{lit.}\right]/\left[\frac{g.}{lit.}\right]\left[min.\right]$	
		Observed	Calculated
D-Glucose D-Xylose	0.05 0.50	$2.4\times10^{-5.0}$	$2.4\times10^{-5.0}$
D-Glucose D-Xylose	0.05 0.25	2.6 ,,	2.7 ,,
D-Glucose D-Xylose	0.05 0.10	3.1 ,,	3.1 ,,
D-Glucose D-Xylose	0.05 0.033	3.7 ,,	3.6 ,,
D-Glucose D-Xylose	0.05 0.0167	3.9 ,,	3.9 ,,
D-Glucose D-Xylose	0.05 0.01	4.1 ,,	4.0 ,,
D-Glucose D-Xylose	0.01 0.025	2.3 ,,	2.4 ,,
D-Glucose D-Xylose	0.01 0.0167	2.5 ,,	2.5 ,,
D-Glucose D-Xylose	0.005 0.025	2.0 ,,	2.1 ,,
D-Glucose D-Xylose	0.005 0.0167	2.2 ,,	2.0 ,,

Also in this case the calculated values of $\phi_{a,b}$ agreed well with observed ones (see Table III). These facts may be taken as the evidence that D-glucose and D-xylose act competitively with each other.

(b) *D-Galactose vs. D-Xylose*—Experiments similar to the foregoing were carried out with D-galactose (S_a) and D-xylose (S_b) using 2,6-dichlorophenol-indophenol as hydrogen acceptor. Using the same hydrogen acceptor, the following values of V_m/ε and M have previously been determined for the two substrates.

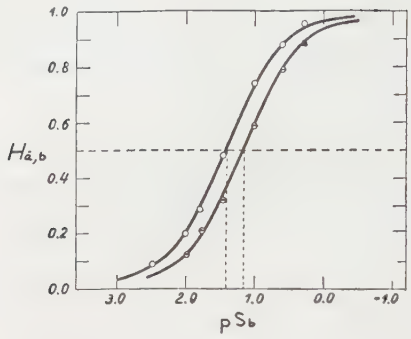
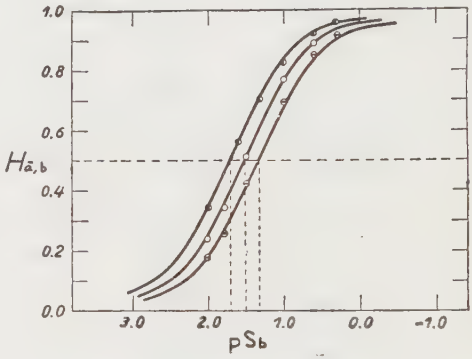


FIG. 1. The inhibition-pS$_b$-curve of D-xylose.

Hydrogen-acceptor: thionine. Ordinate: the degree of inhibition caused by D-xylose. Abscissa: $-\log_{10}$[D-xylose]. ⊖: Observed value in the presence of 0.05*mole/lit.* D-glucose. ○: Observed value in the presence of 0.025 *mole/lit.* D-glucose.

FIG. 2. The inhibition-pS$_b$-curve of D-xylose.

Hydrogen-acceptor: 2,6-Dichlorophenol-indophenol. ⊖: Observed value in the presence of 0.050 *mole/lit.* D-glucose. ○: Observed value in the presence of 0.025 *mole/lit.* D-glucose. ◐: Observed value in the presence of 0.50 *mole/lit.* D-galactose.



$$V_m^{(a)}/\varepsilon = 1.30\times10^{-5\cdot0}\left[\frac{mole}{lit.}\right]\Big/\left[\frac{g.}{lit.}\right]\left[min.\right]$$ For D-galactose

$$M^{(a)} \quad \left[\frac{mole}{lit.}\right]$$ (H-acceptor: 2,6-dichloro-phenol-indophenol)

$$V_m^{(b)}/\varepsilon = 3.0\times10^{-5\cdot0}\left[\frac{mole}{lit.}\right]\Big/\left[\frac{g.}{lit.}\right]\left[min.\right]$$ For D-xylose

$$M^{(b)} = 0.012 \quad \left[\frac{mole}{lit.}\right]$$ (H-acceptor: 2,6-dichloro-phenol-indophenol.)

Applying these values to Eq. (6) the rates of the reaction ($v_{\bar{a},b}/\varepsilon$) to be observed in the simultaneous presence of the two sugars were calculated and they were compared with the values obtained by experiment. The results presented in Table II show that the agreement between calculated and observed values was excellent.

The relationship between $H_{\bar{a},b}$ and pS$_b$ is illustrated in Fig. 2. The value of $\phi_{\bar{a},b}$ obtained from this curve was 0.020 *mole/lit.* at [S$_a$]=0.50 *mole/lit.* This figure coincided again satisfactorily with that calculated

TABLE II

The Rate of the Reaction ($v_{a,b}/\varepsilon$) in the Simultaneous Presence of D-Galactose (S_a) and D-Xylose (S_b)

H-Acceptor: 2,6-Dichlorophenol-indophenol. Temperature: 30°. pH: 7.2.

Substrates	Concentrations of substrates (*mole/lit.*)	Rate $(v_{a,b}/\varepsilon)\left[\frac{mole}{lit.}\right]/\left[\frac{g.}{lit.}\right]\left[min.\right]$	
		Observed	Calculated
D-Galactose D-Xylose	0.50 0.50	$3.0\times10^{-5.0}$	$3.1\times10^{-5.0}$
D-Galactose D-Xylose	0.50 0.25	3.1 ,,	3.2 ,,
D-Galactose D-Xylose	0.50 0.10	3.4 ,,	3.4 ,,
D-Galactose D-Xylose	0.50 0.05	3.7 ,,	3.7 ,,
D-Galactose D-Xylose	0.50 0.025	4.0 ,,	4.0 ,,
D-Galactose alone	0.50	5.0 ,,	5.4 ,,

TABLE III

The $\phi_{\bar{a},b}$ Values of the Inhibition Caused by D-Xylose

Temperature: 30°. pH: 7.2.

H-Acceptor	Substrates ($S_{\bar{a}}$)	Concentration (*mole/lit.*)	Michaelis constant (*mole/lit.*)		$\phi_{\bar{a},b}$ (*mole/lit.*)	
			$M^{(a)}$	$M^{(b)}$	Obs.	Calc.
Thionine	D-Glucose	0.050	0.0085	0.0105	0.073	0.073
Thionine	D-Glucose	0.025	0.0085	0.0105	0.040	0.042
2,6-Dichloro-phenol-indophenol	D-Glucose	0.050	0.0150	0.0120	0.050	0.052
2,6-Dichloro-phenol-indophenol	D-Glucose	0.025	0.0150	0.0120	0.031	0.032
2,6-Dichloro-phehol-indophenol	D-Galactose	0.50	0.72	0.0120	0.020	0.020

from Eq. (10) (see Table III). It may, therefore, be concluded that D-galactose and D-xylose also act competitively with each other:

II. *Effect of Non-Oxidizable Sugars on the Action of the Enzyme*—As was previously shown, our enzyme cannot oxidize D-arabinose, L-rhamnose and α-methyl-glucoside. Among these substances D-arabinose was found to exert an inhibitory effect upon the action of the enzyme on oxidizable sugars (glucose, galactose and mannose). Fig. 3 shows the *H*-pG-curve obtained for galactose using 2, 6-dichlorophenol-indophenol as hydrogen acceptor. Similar curves —corresponding to Eq. (12)—were also obtained with other oxidizable sugars investigated. The ϕ-values determined by these curves were found to be in good agreement with theoretical ones calculated by Eq. (13) assuming K_G to be 0.48 *mole/lit.* (Table IV). This coincidence indicates that D-arabinose hinders the enzyme reaction by competing with the substrate for the enzyme molecule.

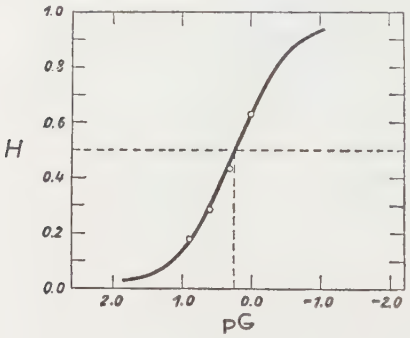


FIG. 3. The inhibition-pG-curve of D-arabinose.

Hydrogen-acceptor: 2, 6-dichlorophenol-indophenol. ○ : observed value in the presence of 0.125 *mole/lit.* D-galactose.

TABLE IV

The ϕ-Value of the Inhibition Caused by D-Arabinose

H-Acceptor: 2, 6-Dichlorophenol-indophenol. The theoretical ϕ-values were calculated by assuming $K_G = 0.48$ *mole/lit.* Temperature: 30°. pH: 7.2.

Substrate (S_a)	Concentrations of substrates (*mole/lit.*)	Michaelis constant $M^{(a)}$ (*mole/lit.*)	ϕ (*mole/lit.*)	
			Observed	Calculated
D-Glucose	0.010	0.015	0.80	0.80
D-Galactose	0.125	0.72	0.56	0.56
D-Mannose	0.250	1.09	0.60	0.59

There was no indication that the action of the enzyme upon oxidizable sugars was interfered with to any measurable extent by the presence of L-rhamnose or α-methyl-glucoside. This may be due to the small affinity of these substances to the enzyme molecule.

III. *Effect of 6-Phospho-D-Glucose upon the Oxidation of D-Glucose*— As described previously (*3, 4*), 6-phospho-D-glucose can be oxidized by our enzyme presparation. The Michaelis constant was found to be $M=0.001$ *mole/lit.* (H-acceptor: 2,6-dichlorophenol-indophenol), a value that is 15 times smaller than that of glucose ($M=0.015$ *mole/lit.* H-acceptor: 2,6-Dichlorophenol-indophenol). In an attempt to determine whether D-glucose (S_a) and 6-phospho-D-glucose (S_b) act competitively with each other, the rates of the oxidation of 0.01 *mole/lit.* D-glucose in the presence and absence of 0.025 *mole/lit.* 6-phospho-D-glucose were compared using 2,6-dichlorophenol-indophenol as hydrogen acceptor. If the two substances act competitively, we may expect from Eq. (10) that the oxidation of glucose will be inhibited as much as 93 per cent by the effect of 0.025 *mole/lit.* 6-phospho-D-glucose. The results of experiment showed, however, that the rate of glucose oxidation was not affected at all by the presence of the phosphoric ester. It may, therefore, be suspected that the phosphoric ester might have been dealt with by some specific enzyme that was present as an impurity in our preparation of glucose dehydrase.

SUMMARY

1. Using the glucose dehydrogenase isolated from *Aspergillus oryzae* the mutual actions between two oxidizable substrates or between oxidizable and non-oxidizable substances were investigated by kinetic methods.

2. Competitive actions were found to take place between D-glucose, D-xylose, D-galactose, D-mannose and D-arabinose, among which only D-arabinose has been shown to be innert against the action of the enzyme. The mutual effects expected to occur between two substrates were calculated from the Michaelis constants and the maximum velocities of oxidation (to be observed in the presence of sufficiently high concentrations of substrate) which had been determined separately for each substrate, and the calculated values were shown to be in good agreement with the observed ones.

3. 6-Phospho-D-glucose which has been shown to be dehydrogenated —with a fairly small Michaelis constant—by the enzyme preparation used, did not interfere with the oxidation of D-glucose by the enzyme. It was, therefore, suspected that the oxidation of this substance was caused by some specific enzyme that was present as an impurity in the preparation of the glucose dehydrase.

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